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Immunomodulation and Alterations in Tumor Cell Adhesion as Potential Anticarcinogenic Mechanisms of Conjugated Linoleic Acid

by

Daena Christine Winchell ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science

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"Finally, brothers, whatever is true, whatever is noble, whatever is right, whatever is pure, whatever is lovely, whatever is admirable- if anything is excellent or praiseworthy- think about such things."

Phillipians 4:8 (New International Version)

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Immunomodulation and Alterations in Tumor Cell Adhesion as Potential Anticarcinogenic Mechanisms of Conjugated Linoleic Acid* by *Daena Christine Winchell* in partial fulfillment of the requirements for the degree of *Masters of Science* in *Nutrition and Metabolism.*

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Catherine J. Field

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Lawrence H. Wang

Date of Committee Approval: March 29, 1999

This is dedicated to my brothers,
Marty- for making me laugh and for your passion for life
Neil- for your creativity and for your courage in following your heart
and to my sister,
Jan- for being the best sister and friend a person could ever ask for.

Abstract

Anticarcinogenic effects of conjugated linoleic acid (CLA) have been shown however mechanisms are unknown. The objective of these studies was to examine the effects of CLA on the immune system and on cell-extracellular matrix (ECM) adhesion as potential anti-tumor mechanisms. Feeding rats 1% w/w CLA in a diet for 21 d with a similar lipid composition to that consumed by humans (15% w/w lipid, polyunsaturated/saturated (P/S) fat ratio= 1.0 or 0.2) increased ($p=0.05$) splenocyte proliferation, particularly in rats fed a low P/S diet. CLA fed in a low P/S diet increased ($p<0.05$) the proportion of immune cells that were activated after mitogen stimulation. CLA was incorporated into adipose and immune cell membrane lipid in rats fed the low P/S diet, suggesting competition between CLA and linoleic acid (LA). *In vitro*, CLA reduced tumor cell proliferation and was incorporated into the PC and PE fractions ($p=0.0001$) but did not significantly change cell-ECM adhesion or expression of integrins. The isomer distribution of CLA in lipids differed between lipid fractions and between host and tumor cells. These results support a positive effect of dietary CLA, when added to a low P/S diet on immune function. Although CLA is readily incorporated into tumor membrane lipids, it does not reduce tumor growth through effects on cell adhesion.

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Table of Contents

Page

Chapter One- Literature Review

I. Conjugated Linoleic Acid- What Is It? 1
II. Physiological Effects of CLA 4
III. Anticarcinogenic Mechanisms of CLA 4
  A) Preventing Interactions Between Carcinogens and Target Cells 5
  B) Effects on Neoplastic Development 6
  C) Alterations in Cell Adhesion 7
  D) Fatty Acid Absorption and Metabolism 8
  E) Actions as an Antioxidant 11
  F) Changes in Membrane Composition 13
    i) Incorporation into Various Tissues 15
    ii) Incorporation into Tumor Cells 17
    iii) Incorporation into Immune Cells 18
  G) Eicosanoid Production 18
  H) Actions on Anti-tumor Immune Defenses 20
    i) CLA and Immune Function 22
    ii) Dietary Fat and Immune Function 23
IV. Directions for Future Research 24
V. References 25

Chapter Two- Study Rationale

I. Introduction 38
II. Hypotheses and Objectives 39
III. References 40

Chapter Three- Conjugated Linoleic Acid and Immune Function in Healthy Rats

I. Introduction 43

II. Materials and Methods 44

- *A) Experimental Design* 44
- *B) Lymphocyte Isolation* 45
- *C) Natural Killer Cytotoxicity* 46
- *D) Splenocyte Phenotyping* 47
- *E) Splenocyte Proliferation* 48
- *F) Interleukin-2 Production* 49
- *G) Interferon Gamma Production* 50
- *H) Tumor Necrosis Factor Alpha Production* 51
- *I) Nitric Oxide Production* 52
- *J) Adipose Triglyceride Analysis* 53
- *K) Splenocyte Phospholipid Analysis* 55
- *L) Body Composition* 57
- *M) Statistical Analyses* 59

III. Results 60

- *A) Body Weight* 60
- *B) Food Intake* 61
- *C) Spleen Weight* 62
- *D) Epidydymal Fat Pad Weight* 63
- *E) Natural Killer Cytotoxicity* 64
- *F) Splenocyte Phenotyping* 65
  - *i) Freshly Isolated Splenocytes* 65
  - *ii) Splenocytes Stimulated with Concanavalin A and Cultured for 48 Hours* 67
- *G) Proliferation* 70
  - *i) Unstimulated Splenocytes* 70
  - *ii) Concanavalin A- stimulated Splenocytes* 72
  - *iii) PMA + Iono-stimulated Splenocytes* 73

H) Interleukin-2 Production 73
I) Nitric Oxide Production 75
J) Interferon Gamma Production 76
K) Tumor Necrosis Factor Alpha Production 78
L) Fatty Acid Composition of Epidydymal Fat Pad Triglycerides 80
M) Splenocyte Phospholipid Fatty Acid Analysis 82
N) Body Composition 84
IV. Discussion 88
A) Food Intake and Body Weight 88
B) CLA and Immune Function 92
i) Mitogen-Induced Proliferation 92
ii) Characterization of Immune Cells in Spleen 94
iii) Natural Killer Cell Activity 95
iv) Nitric Oxide Production 96
v) IL-2 Production 97
vi) IFN-γ Production 98
vii) TNF-α Production 99
C) CLA Incorporation into Tissues 100
i) Adipose Triglycerides 100
ii) Stimulated Splenocyte Phospholipid Fractions 103
V. Summary 106
VI. References 107

Chapter Four- Conjugated Linoleic Acid and Tumor Cell Adhesion

I. Introduction 114
II. Materials and Methods 115
A) Cell Line Maintenance 115
B) Cell Proliferation 115
C) Cell Adhesion Assay 116

- D) Tumor Cell Phenotyping 117
- E) Tumor Phospholipid Analysis 121
- F) Statistical Analysis 122

III. Results 123

- A) Cell Proliferation 123
- B) Cell Adhesion 125
- C) Cell Phenotyping 127
- D) Tumor Cell Phospholipid Analysis 129

IV. Discussion 133

- A) Tumor Cell Proliferation 133
- B) Tumor Cell Phospholipid Analysis 135
  - i) Phosphatidylcholine Fraction 136
  - ii) Phosphatidylethanolamine Fraction 137
  - iii) CLA Isomer Distribution in Phospholipid Fractions 138
  - iv) Lipid Summary 139
- C) Tumor Cell Adhesion 140
- D) Tumor Cell Integrin Expression 141

V. Summary 143

VI. References 144

Chapter Five- General Summary and Discussion

I. General Summary 150

- A) Study One 150
- B) Study Two 151

II. Integration of the Diet Study and Cell Culture Work 152

III. Directions for Future Research 154

IV. Practical Implications 155

V. References 157

Appendix A. Anti-Tumor Immune Defenses 159

Appendix B. Diet Compositions for Experiment One 160
Appendix C. Compositions of Various Solutions 163
Appendix D. Calculations to Determine Carcass Body Composition 165
Appendix E. Rationale for Study Design of Cell Culture Work 168

List of Tables

Page

Chapter One

Table 1.1 *The Physiological Effects of CLA as Determined in Animal Models and In Vitro Studies* *4*

Table 1.2 *Alterations in Cell Proliferation Due to Dietary CLA or In Vitro CLA Incubation* *6*

Table 1.3 *Classification of the Major Cell Adhesion Molecules* *8*

Table 1.4 *Current Literature Regarding the Antioxidant Activity of CLA* *12*

Chapter Three

Table 3.1 *Monoclonal Antibodies Utilized in Phenotyping* *48*

Table 3.2 *Phenotypes of Freshly Isolated Splenocytes* *65*

Table 3.3 *CD4 and CD8 cells that are CD5 positive in Freshly Isolated Splenocytes* *66*

Table 3.4 *Phenotypes of Splenocytes Cultured for 48 Hours and Stimulated with Concanavalin A* *67*

Table 3.5 *Phenotypes of Splenocytes Stimulated with Concanavalin A, Cultured for 48 Hours and Expressing the Transferrin Receptor (ox26+)* *68*

Table 3.6 *Phenotypes of Splenocytes Stimulated with Concanavalin A, Cultured for 48 Hours, and Expressing the Interleukin-2 Receptor (ox39+)* *69*

Table 3.7 *Fatty Acid Profile of Male Epididymal Fat Pad Triglycerides as Determined by Gas Liquid Chromatography* *80*

Table 3.8 *Each Isomer as a Percent of Total CLA in Epididymal Fat Pads From Rats fed CLA-supplemented Diets* *81*

Table 3.9 *Fatty Acid Profile of the Phosphatidylcholine Fraction of Splenocytes Stimulated for 48 Hours with Concanavalin A as Determined by Gas Liquid*

Chromatography 82

Table 3.10 Fatty Acid Profile of the Phosphatidylethanolamine Fraction of Splenocytes Stimulated for 48 Hours with Concanavalin A as Determined by Gas Liquid Chromatography 83

Table 3.11 Polyunsaturated Fatty Acid Composition of Diet and Adipose Triglycerides for Animals fed Low P/S Diets as Determined by Gas Liquid Chromatography 102

Chapter Four

Table 4.1 Mean Fluorescence of MDA-MB 231 Cells Expressing CD29 or CD104 Following 48 Hour Incubation with Various Fatty Acids 127

Table 4.2 Fatty Acid Analysis of the Phosphatidylcholine Fraction of MDA-MB 231 Cells Following 48 Hour Incubation with Various Fatty Acids 129

Table 4.3 Fatty Acid Analysis of the Phosphatidylethanolamine Fraction of MDA-MB 231 Cells Following 48 Hour Incubation with Various Fatty Acids 131

Table 4.4 Conjugated Linoleic Acid Isomer Analysis of the Phosphatidylcholine and Phosphatidylethanolamine Fractions of MDA-MB 231 Cells Following 48 Hour Incubation with 60 μM each of Linoleic and Conjugated Linoleic Acid 132

Appendices

Table B.1 Fatty Acid Profile of Experimental Diets 161

List of Figures

Page

Chapter One

Figure 1.1. *Conjugated Linoleic Acid (c9,t11 isomer)* 3

Figure 1.2. *Potential sites for CLA to inhibit tumorigenesis in cancer development* 5

Figure 1.3. *Structure of a Phospholipid Bilayer* 14

Figure 1.4. *Biosynthetic Pathways for Eicosanoid Production* 18

Chapter Three

Figure 3.1 *Diet Design* 45

Figure 3.2 *GLC Expansion of CLA Isomer Peaks* 55

Figure 3.3 *Body Weight Measurement During the Feeding Period* 60

Figure 3.4 *Dietary Intake During the Feeding Period* 61

Figure 3.5 *Spleen Weight Per Body Weight* 62

Figure 3.6 *Epidydymal Fat Pad Weight Per Body Weight* 63

Figure 3.7 *Natural Killer Cytotoxicity at Various Effector: Target Ratios* 64

Figure 3.8 *^{3}H-Thymidine Incorporation into Unstimulated Cultured Splenocytes* 70

Figure 3.9 *Splenocytes Cultured for 42 Hours with or without Concanavalin A Stimulation* 71

Figure 3.10 *^{3}H-Thymidine Incorporation into Cultured Splenocytes Stimulated with Concanavalin A* 72

Figure 3.11 *^{3}H-Thymidine Incorporation into Cultured Splenocytes Stimulated with Phorbol Myristate Acetate + Ionomycin* 73

Figure 3.12 *Interleukin-2 Production by Splenocytes Stimulated with Concanavalin A for 48 hours* 74

Figure 3.13 *Nitric Oxide Production by Unstimulated Splenocytes* 75

Figure 3.14 *Nitric Oxide Production by Splenocytes Stimulated with Concanavalin A for 48 hours* 76

Figure 3.15 *Interferon Gamma Production by Splenocytes Stimulated with Concanavalin A for 48 hours* 77

Figure 3.16 *Tumor Necrosis Factor Alpha Production by Unstimulated Splenocytes* 78

Figure 3.17 *Tumor Necrosis Factor Alpha Production by Splenocytes Stimulated with Concanavalin A for 48 Hours* 79

Figure 3.18 *Ratio of* c9*t*11:*t*10c12 *Isomers in CLA-Supplemented Rat Adipose Triglycerides versus Diet* 81

Figure 3.19 *Percentage of Total Body Composition as Water* 85

Figure 3.20 *Percentage of Total Body Composition as Protein* 86

Figure 3.21 *Percentage of Total Body Composition as Lipid* 87

Figure 3.22 *Percentage of Total Body Composition as Ash* 88

Chapter Four

Figure 4.1 *Forward Scatter (Size) versus Side Scatter (Granularity) of MDA-MB 231 Cells to Allow Elucidation and Gating of the Viable Cell Population* 119

Figure 4.2 *Binding of Fluoroscein isothiocyanate-Conjugated Goat Anti-mouse* $IgG_{1,\lambda}$ *to MDA-MB 231 Cells to Determine Background Fluorescence* 119

Figure 4.3 *CD29+ Binding versus Binding of Fluoroscein Isothiocyanate-Conjugated Goat Anti-mouse* $IgG_{1,\lambda}$ *to MDA-MB 231 Cells* 120

Figure 4.4 *CD104+ Binding versus Binding of Fluoroscein Isothiocyanate-Conjugated Goat Anti-mouse* $IgG_{1,\lambda}$ *to MDA-MB 231 Cells* 120

Figure 4.5 Regions Used in Analysis of CD104+ Binding to MDA-MB 231 Cells 121

Figure 4.6 Proliferation of MDA-MB 231 Cells After a 48 Hour Incubation With Various Fatty Acids 123

Figure 4.7 Proliferation of MDA-MB 231 Cells After a 72 Hour Incubation With Various Fatty Acids 124

Figure 4.8 Adhesion of MDA-MB 231 Cells to Collagen IV After a 48 Hour Incubation with Various Fatty Acids 125

Figure 4.9 Adhesion of MDA-MB 231 Cells to Fibronectin After a 48 Hour Incubation with Various Fatty Acids 126

Figure 4.10 Adhesion of MDA-MB 231 Cells to Matrigel After a 48 Hour Incubation with Various Fatty Acids 127

Appendices

Figure B.1 Major Lipid Types in Experimental Diets 162

Figure B.2 CLA Isomeric Composition in Supplemented Diets 162

Figure D.1 Percent of Body Water Versus Percent of Body Lipid for Male Rats 166

Figure E.1 Concentration Curve for the Effects of Palmitic Acid on Proliferation of MDA-MB-231 Tumor Cells 168

Figure E.2 Proliferation of MDA-MB 231 Cells in Various Cell Culture Conditions 169

Figure E.3 Concentration Curve for the Effects of Linoleic Acid on Proliferation of MDA-MB-231 Tumor Cells 170

Abbreviations

AA-	arachidonic acid
ANSA-	8-anilino-1-naphthalene sulfonic acid
AOAC-	Association of Official Analytical Chemists
BHT-	butylated hydroxytoluene
BP-	benzo(a)pyrene
BrdU-	bromodeoxyuridine
BSA-	bovine serum albumin
CEA-	carcinoembryonic antigen
CLA-	conjugated linoleic acid
Con A-	concanavalin A
CTL-	cytotoxic T lymphocyte
DHA-	docosahexaenoic acid
DM-	dry matter
DMBA-	dimethylbenz(a)anthracene
DNA-	deoxyribonucleic acid
ECM-	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EPA-	eicosapentaenoic acid
FAME-	fatty acid methyl ester
FCS-	fetal calf serum
FITC-	fluorescein isothiocyanate
GC-	gas chromatography
GLC-	gas liquid chromatography
HETE-	hydroxy eicosatetraenoic acid
HMEC-	human mammary epithelial cell
ICAM-	intercellular adhesion molecule
IFN-γ-	interferon gamma
IL-2	interleukin-2
INDO-	indomethacin
IQ-	2-amino-3-methylimidazo[4,5-f]quinoline
LA-	linoleic acid
LNA-	linolenic acid
LPS-	lipopolysaccharide
LT-	leukotriene
MCA-	mucin-like carcinoma associated antigen
MDA-	malondialdehyde
ME-	methyl ester
MHC-	major histocompatibility
MNU-	methylnitrosourea
NDGA-	nordihydroguaiaretic acid
NK-	natural killer cells
NO-	nitric oxide
PBS-	phosphate buffer solution

PC-	phosphatidylcholine
PE-	phosphatidylethanolamine
PE-	phycoerythrin
PG-	prostaglandin
PHA-P	phytohemagglutinin
PI-	phosphatidylinositol
PKC-	protein kinase C
PL-	phospholipid
PLPC-	1-palmitoyl-2-linoleoyl phosphatidylcholine
PMA + Iono-	phorbol myristate acetate + ionomycin
P/S ratio-	polyunsaturated/saturated fat ratio
PS-	phosphatidylserine
PUFA-	polyunsaturated fatty acid
PWM-	pokeweed mitogen
RNA-	ribonucleic acid
SM-	sphingomyelin
TAA-	tumor-associated antigen
TBARS-	thiobarbituric acid-reactive substance
T_{CTL}-	cytotoxic T-cell
TG-	triacylglycerol/ triglyceride
T_H-	helper T-cell
TLC-	thin layer chromatography
TNF-α-	tumor necrosis factor alpha
TPA-	12-O-tetradecanoylphorbol-13-acetate
TPA-	tumor polypeptide antigen
TX-	thromboxane
VCAM-	vascular cell adhesion molecule

Chapter One- Literature Review

I. Conjugated Linoleic Acid- What Is It?

Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of linoleic acid (LA, *c*9*c*12-18:2). Its conjugated double bonds are typically located at the ninth and eleventh or the tenth and twelfth carbon atoms and all *cis-* and *trans-* isomers are possible. More recently, it has been determined that double bonds at the eighth and tenth or eleventh and thirteenth carbon atoms can be found in commercial preparations of CLA (Sehat *et al.*, 1998) and some French cheeses (Lavillonnière *et al.*, 1998). Also, Rickert *et al.* (1999) were able to resolve *t*6*t*8 and *t*13*t*15 isomers in cheese. Thus, there are potentially thirty-two or more isomers of CLA. Four of these (*c*9,*t*11-, *t*10,*c*12-, *t*9,*t*11-, and *t*10,*t*12- 18:2) account for more than 90% of CLA identified in foods and commercial mixtures (Ha *et al.*, 1989). Of all isomers, the most biologically active form is believed to be *c*9,*t*11 (see ***Figure 1.1***) which is formed from LA in the rumen stereospecifically by *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966) and is the isomer that is reported to be incorporated into membrane phospholipids (PL) in mouse forestomach (Ha *et al.*, 1990; Benjamin *et al.*, 1990), and in rat liver and mammary tumor (Ip *et al.*, 1991). It has also been shown to be the naturally occurring isomer in meat and dairy products (Ma *et al.*, in press), however one group also found other minor isomers in these food products (Ha *et al.*, 1989).

CLA is present in the diet. The average American consumption of this fatty acid is estimated at several hundred milligrams per person per day (Shultz *et al.*, 1992A). However, considering the variation in CLA content of food products (to be discussed shortly), these estimates are questionable. Canadian consumption levels are estimated at approximately 100 mg/day (Ens, submitted) CLA has been reported to exist in ruminant meats (eg. beef, lamb) at estimated levels of 3.1 - 8.5 mg/g fat. CLA levels in meats have been suggested to be modifiable by diet (Shantha *et al.* 1997; Jahreis *et al.*, 1997; Jiang *et al.*, 1996). CLA has also been found in various dairy products although reported levels differ. Whereas Ma *et al.* (in press) estimated CLA levels in milk to be 1.8- 5.5 mg/g fat, Jiang *et al.* (1996) reported much more variable levels of 2.5-17.7 mg/g fat.

Likewise, there is variation in reported levels in cheeses. Lin *et al.* (1998) found that vacuum pouch packed cheese contained only 2.7 mg/g fat while Garcia-Lopez and colleagues (1994) determined process cheese to contain 10.7 mg/g fat. Lavillonnière *et al.* (1998) found that some French cheeses contained as much as 15.8 mg/g fat. Such variation in CLA levels may be due to several factors including carbon-centered free-radical type oxidation of linoleic acid (Cawood *et al.*, 1983), heat treatments such as pasteurization or grilling (Ha *et al.*, 1989), aging processes and protein quality (Shantha and Decker, 1993; Garcia-Lopez *et al.*, 1994) and production practices (Jahreis *et al.*, 1997; Dhiman *et al.*, 1996,1997). Surprisingly, CLA levels in turkey are similar to those in ruminant products (Chin *et al.*, 1992), perhaps due to hindgut fermentation or intestinal flora, although this is not known. CLA is found mainly in the triglyceride (TG) form in these dietary sources. Non-ruminant sources such as chicken and eggs have lower quantities (0.6 - 0.9 mg/g fat) (Decker, 1995) and are poorer sources along with plants and seafood.

In ruminant animals, CLA appears to be produced predominantly as an intermediate in the bioisomerization of LA to *trans* vaccenic acid (*t*11- 18:1) by the rumen anaerobe *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966). The presence of CLA in non-ruminant animals appears to be primarily through consumption of ruminant products. However, Chin *et al.* (1994B) found that normal rats fed 5% w/w LA (but CLA-free) diet had body CLA concentrations that were five to ten-fold higher than comparable germ-free rats. An increase in CLA levels was only seen when LA was administered in free fatty acid (FFA) form and not with LA esterified to TG. Herbel *et al.* (1998) found that feeding humans safflower oil which contains TG-esterified LA also did not increase plasma CLA levels. Administering LA in FFA form was not studied. These two studies suggest that non-ruminants may also possess the ability to biosynthetically produce CLA in small amounts, perhaps via intestinal flora, when diets high in free LA are fed.

In humans, CLA is likely present largely due to dietary consumption. Huang *et al.* (1994) demonstrated that plasma CLA levels correlated with ingestion of cheddar cheese. This indicates a strong dietary contribution to human CLA levels. In addition, Salminen et al (1998) determined that subjects fed diets high in *trans* fatty acids had increased CLA in serum lipids. This group also suggested that their results did not support conversion of LA to CLA as all diet treatments contained similar levels of LA, yet different serum lipid CLA levels were seen. It was not indicated whether LA was administered in FFA or TG form which may have affected the potential microbial conversion or metabolism.

Together, these studies suggest that there may be two main sources for the endogenous presence and synthesis of CLA. However, in humans and monogastric animals consuming diets with physiological levels of LA, evidence indicates that dietary intake of CLA is quantitatively greater with a minor , if any, contribution from endogenous synthesis contributing to the total body pool.

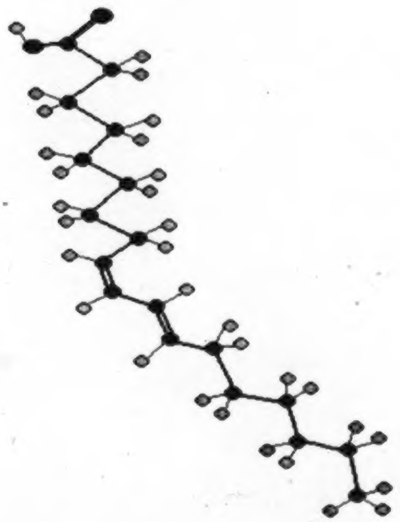

Figure 1.1. Conjugated Linoleic Acid (c9,t11 isomer)

II. Physiological Effects of CLA

Physiological Effect	Reference
Anti-atherogenic	Lee *et al*., (1994) Nicolosi *et al*., (1997)
Metabolic	Park *et al*., (1997) Dugan *et al*., (1997)
Immunomodulatory	Miller *et al*., (1994) Wong *et al*., (1997) Chew *et al*., (1997) Sugano *et al*., (1998) Hayek *et al*., (1999)
Anti-diabetogenic	Houseknecht *et al*. (1998)
Anti-carcinogenic	Pariza and Hargraves, (1985) Ha *et al*., (1990) Liew *et al*., (1995) Ip *et al*., (1991) Visonneau *et al*., (1997) Cesano *et al*., (1998)

Table 1.1. The Physiological Effects of CLA as Determined in Animal Models and In Vitro Studies

CLA has been shown to exert several physiological effects (see Table 1.1) Anti-carcinogenic effects of CLA have been reported in several animal models of cancer including mouse epidermal (Pariza *et al*., 1986), mouse forestomach (Ha *et al*., 1990), rat colon (Liew *et al*., 1995) and rat mammary (Ip *et al*., 1991) tumorigenesis. These studies reported a decrease in the incidence and multiplicity of tumors when CLA was fed at levels as low as 0.1% (w/w). The anti-tumorigenic effects of CLA will be the focus of the proceeding discussion.

III. Anticarcinogenic Mechanisms of CLA

The mechanisms whereby CLA reduces tumorigenesis remain to be elucidated. CLA could potentially exert its actions at various points in the time dimension of tumor development. That is, CLA may be anticarcinogenic due to actions prior to or during tumor initiation, promotion, or progression. This is summarized in ***Figure 1.2***.

HOW CLA MIGHT ACT AS AN INHIBITOR	**SEQUENCE LEADING TO NEOPLASIA**
	Precursor compounds
	⇓
Preventing formation of carcinogen	--------------------------------
	⇓
	Carcinogenic Compounds
	⇓
Blocking Agent	--------------------------------
	⇓
	Reactions with Cellular Targets
	⇓
Suppressing Agent	--------------------------------
	⇓
	Neoplastic Manifestations

Figure 1.2. Potential sites for CLA to inhibit tumorigenesis in cancer development (Modified from Wattenberg, 1985)

As a lipid and, more specifically, a fatty acid, there are several mechanisms through which CLA might act to elicit its anticarcinogenic effects.

A) Preventing Interactions Between Carcinogens and Target Cells

CLA could potentially act either by preventing a pro-carcinogen from being converted to its active form, or by enhancing the activity of enzymes that detoxify carcinogens already in their active form.

Studies suggest that CLA may affect carcinogens in three primary ways. Through a general micelle trapping effect, as demonstrated in several fatty acids (Hayatsu *et al.* 1988; Liew *et al.* 1995), CLA may trap the mutagen, thus preventing the induction of carcinogenesis. Micelle trapping was also effective when tested with other fatty acids such as LA and oleic acid (Hayatsu *et al.* 1988), indicating that this effect is not unique to CLA. Also, micelle trapping was not as evident for CLA (Liew *et al.* 1995), despite an inhibition in tumorigenesis suggesting additional sites of action of CLA in inhibiting tumor development.

CLA may also reduce carcinogen binding to DNA or lipids (Liew *et al.*, 1995), although evidence exists to the contrary (Ip *et al.*, 1994B). Such contrasting results suggest that CLA may affect different carcinogens in varying ways. Thirdly, CLA has been reported to inhibit enzyme activation so that procarcinogens are converted to their active form less rapidly (Ha *et al.*, 1987; Liew *et al.*, 1995). Finally, CLA could also enhance detoxification of carcinogens as a means of reducing tumor incidence. However, research has not supported such a mechanism (Ip *et al.*, 1991; Ip *et al.*, 1994B).

Although there is some indirect evidence that CLA affects carcinogens, it does not appear to be the primary anti-carcinogenic mechanism of CLA.

B) Effects on Neoplastic Development

Alterations in Proliferation / Apoptosis	
Mammary Cells	
-reduced epithelial cell proliferation	Cunningham *et al.*, (1997)
-altered gland development resulting in chemoprotective effect	Ip *et al.*, (1994); Ip *et al.* (1994A); Ip *et al.*, (1995); Thompson *et al.*, (1997)
Tumor Cells	
-reduced proliferation in several tumor cell lines including mammary	Seaman *et al.*, (1992); Shultz *et al.*, (1992A), (1992B); Schønberg and Krokan, (1995); Yoon *et al.*, (1997); Cornell *et al.*, (1997)
Immune Cells	
-increased mitogen-induced proliferation	Cook *et al.*, (1993); Miller *et al.*, (1994); Wong *et al.*, (1997); Chew *et al.*, (1997)

Table 1.2. Alterations in Cell Proliferation Due to Dietary CLA or In Vitro CLA Incubation

Although it has yet to be studied, CLA could act by enhancing tumor cell apoptosis. Studies examining the effects of ω-3 fatty acids have reported an increase in tumor cell apoptosis both *in vivo* (Calviello *et al.*, 1998) and *in vitro* (Lai *et al.*, 1996). As ω-3 fatty acids have also been shown to reduce tumorigenesis, it is plausible that CLA utilizes a similar mechanism and increases apoptosis of neoplastic cells.

C) Alterations in Cell Adhesion

Cells adhere to each other and to the surrounding basement membrane via various adhesion molecules. Therefore, the function and expression of adhesion molecules are of particular interest in examining metastasis- the process whereby tumor cells dislodge from the primary tumor site, travel through the basal lamina into the circulation, extravasate and establish at a secondary site. Two steps in the metastatic process involve cell-to-cell interactions and cell adhesion. The first is the disruption of the interactions between tumor cells so that they may escape from the primary tumor mass. The second process involves the reattaching of these tumor cells to distant sites to establish a secondary tumor.

Unsaturated fatty acids have been shown to affect metastasis. Rose and colleagues (1994) found that linoleic acid increased the lung metastases of human cancer cells in athymic nude mice. Karmali *et al.* (1993) determined that ω-3 fatty acids reduced lung metastases in rats injected with rat mammary adenocarcinoma cells.

Unsaturated fatty acids have also been shown to alter cell adhesion. Dietary lipid level has been found to alter gene expression (Etkind *et al.*, 1995) as have specific fatty acids. For example, Collie-Duguid and Wahle (1996) found that incubating eicosapentaenoic acid (EPA; 18:3(3)) and docosahexaenoic acid (DHA; 22:6(3)) with interleukin-1 beta (IL-1β)-activated human umbilical vein endothelial cells inhibited the expression of intercellular adhesion molecule (I-CAM), vascular adhesion molecule (VCAM-1) and E-selectin. Likewise, Lu *et al.* (1995) showed that EPA modulated ICAM-1 expression. EPA has been shown to reduce adhesion of a human breast cancer cell line to Matrigel, collagen IV, and fibronectin (German and Johanning, 1997). In contrast, EPA and LA were found to increase adhesion of another human mammary adenocarcinoma to Matrigel and collagen IV (Johanning and Lin, 1995). Such changes in gene expression or function of these adhesion molecules are important because modulations in adhesive properties- between tumor cells and between tumor cells and the extracellular matrix- may facilitate or hinder metastasis.

Table 1.3 outlines various cell adhesion molecules and their different adhesive roles.

Family	Constituents	Function
Integrin	Heterodimeric α- and β- subunits	Cell-cell or cell-extracellular matrix adhesion receptors
Cadherin	N-cadherin (neural) P-cadherin (placental) E-cadherin (epithelial)	Calcium-dependent cell-cell adhesion receptors
Selectin	L-selectin (lymphocytes) E-selectin (endothelial) P-selectin (platelets)	Leukocyte-endothelial cell adhesion receptors
Ig superfamily	MHC antigens, CD4, CD8, T cell receptor, N-CAM, Ng-CAM, CSF-1 receptor, PDGF receptor, ICAM-1, VCAM-1, PECAM-1, CEA	Calcium-independent cell-cell adhesion receptors

Table 1.3. Classification of the Major Cell Adhesion Molecules (copied with permission from Johanning (1996))

As ***Table 1.3*** indicates, integrins are of interest in cancer due to their involvement in adhesion with the extracellular matrix. Gui *et al.* (1995) demonstrated this *in vitro* by showing that adding integrin antibodies reduced invasion potential of several tumor cell lines. Integrins also associate with cytoskeletal proteins and have therefore been suggested to be involved in signalling pathways (Schwartz, 1993). Cadherins have also been implicated in tumorigenesis and metastasis with more invasive tumors having a reduction in expression of cadherins (Takeichi, 1993).

Effects on adhesion with polyunsaturated fatty acids (PUFA), particularly PUFA which have been shown to modify tumorigenesis, indicate that CLA may work via a similar mechanism. This remains to be tested by examining both expression and function of adhesion molecules.

D) Fatty Acid Absorption and Metabolism

The absorption of FFA varies considerably depending upon chain length and number of double bonds. For example, fatty acids with twelve carbons or less, or fatty acids with one or more double bonds are easily absorbed whereas longer saturated fatty acids tend to form calcium salts in the digestive tract and are less easily absorbed (Lien *et al.*, 1997). As well, the position of a fatty acid in a TG affects its ability to be absorbed

(Decker, 1996). TG undergo hydrolysis to produce two free fatty acids from the sn-1 and –3 positions of the TG and an sn-2 monoacylglycerol which is readily absorbed. Therefore a longer saturated fatty acid will be more readily absorbed when it is in the sn-2 position compared to sn-1 or –3. Whether or not CLA has a stereospecific incorporation into TG has not been examined, however, theoretically, its incorporation could selectively displace other fatty acids and alter fat absorption and, therefore, energy availability. Potential changes in energy metabolism have not been studied. However, CLA administered as TG and FFA both result in similar mammary tumor inhibition (Ip *et al.*, 1995), suggesting that this is not likely a primary mechanism.

Fatty acids provide most of the calories derived from dietary fat. Energy is derived through the β-oxidation pathway in the mitochondria to produce ATP, CO_2, and H_2O. Saturated and unsaturated fatty acids are oxidized by the same pathway, except that the double bonds present result in less energy being produced (Hunt and Groff, 1990). In situations of increased fatty acid oxidation, an alternate degradative hepatic pathway may also be used to produce ketone bodies- specifically, acetone, β-hydroxybutyrate, and acetoacetate. These substrates are transported from the liver to the peripheral tissues where they are converted to acetyl CoA which can then be metabolized through β-oxidation.

An important concept of lipid oxidation as presented by Jones *et al.* (1985) is that fatty acids are oxidized differently. That is, fatty acids are oxidatively and metabolically unique so that energy production may vary depending on fatty acid composition. As well, as suggested by Yoshida *et al.* (1996), the position of a fatty acid in a TG may also affect lipid metabolism. In their study, EPA and DHA were mainly in the sn-1 and 3 positions in seal oil TG and in the sn-2 position of fish oil TG. It was found that lymph TG structures were similar to that administered orally. However, even when P/S ratios and ω-6/ω-3 ratios were constant, seal oil reduced plasma and liver triglyceride more effectively than fish oil. Such varying metabolic effects with different fatty acids may have implications, especially for tumor and immune cell metabolism, the energy available to these cells, and the metabolites produced.

Lipids have been found to be utilized as an energy source by tumor cells (Argiles, 1988). Various studies have found palmitate and hydroxybutyrate (Mares-Perlman, 1988), acetoacetate (Fenslau, 1975), FFA (Sauer and Dauchy, 1990) and ketone bodies (Sauer and Dauchy, 1983) to be metabolizable by tumor cells. Most notably, Sauer and Dauchy (1990) found LA and arachidonic acid (AA; 20:4(6)) to be taken up by a hepatoma in a dose-dependent manner with 80-85% of the LA being removed after one pass through the hepatoma indicating preferential utilization. Therefore, it would seem reasonable that LA's isomer, CLA, may also be metabolized by the tumor. In addition, the metabolism of CLA may result in the production of various bioactive compounds (discussed below). Such possibilities are still largely unexplored. Liu and Belury (1997) used a radiolabelled tracer study to examine CLA, LA, and AA incorporation into murine keratinocytes. CLA incorporation into phospholipid (PL) classes were similar to that seen for LA with approximately 50% of the radiolabelled CLA and LA being incorporated into the phosphatidylcholine (PC) fraction. When cells were prelabelled with fatty acids, phorbol esters resulted in the release of more CLA compared to LA or AA, however radiolabelled PGE release was considerably lower suggesting that this might be a mechanism of reducing skin tumor promotion. Further experimentation using radiolabelled tracers will prove valuable in future mechanistic studies, particularly if tracers can be used *in vivo* for dietary studies.

While they are not a primary source, lipids are also a fuel for immune cells. Ketone bodies (O'Rourke and Rider, 1989) and fatty acids (Newsholme *et al.*, 1987) have been found to be utilized by various immune cell populations. If CLA were able to act as an energy source and increase the energy available to immune cells, this might serve as a potential mechanism of immunomodulation. Again, this remains to be tested.

Although the metabolism of CLA is not known, CLA appears to alter metabolism as demonstrated by changes in body composition (Cook *et al.*, 1993; Miller *et al.*, 1995; Parks *et al.*, 1997). Pariza *et al.* (1997) determined that epidydymal adipocytes from rats fed 0.5% (w/w) CLA supplemented in their diet demonstrated enhanced lipase activity and norepinephrine-induced lipolysis. This group also found that dietary CLA in mice increased total carnitine palmitoyltransferase activity. These results together suggest that

CLA alters metabolism by reducing fat deposition, increasing adipocyte lipolysis, and enhancing fat oxidation. In contrast, Satory and Smith (1999) found that CLA stimulated lipogenesis and lipid filling in murine preadipocytes. However, this group found a reduction in proliferation of these cells and suggested that this might be the mechanism of reducing overall body lipid. Comparison between the two studies is difficult due to the different cells (adipocyte versus preadipocyte) which were used, however it appears that CLA likely affects lipid metabolism in some way.

E) Actions as an Antioxidant

Oxidation is a consequence of high concentrations of PUFA in membranes. Free radicals, such as the hydroxyl radical or the peroxyl radical scavenge electrons primarily from lipids and nucleic acids which, in the cellular context, lead to damage to the cell membranes and to DNA. Antioxidants end this cycle of free radical production at the expense of being oxidized themselves. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), ß-carotene (precursor of vitamin A) and selenium are dietary antioxidants (Decker, 1995) and CLA may act in a similar fashion. However, from a chemical standpoint, conjugated dienes such as CLA are even more susceptible to oxidation than PUFA with double bonds separated by methylene groups (Banni *et al.*,1998) and are better at scavenging electrons (Zhang and Chen, 1997; Cornelius *et al.*,1991). Although many studies have assumed CLA to have antioxidant properties, the research investigating this possibility is not consistent in its findings (see ***Table 1.4***).

Effects on Lipid Peroxidation	Reference
Mammary and other Somatic Cells	
-antioxidant activity	Ip *et al.*, (1991, 1996)
-no antioxidant activity	Ha *et al.*, (1987)
In vitro Chemical Studies	Ha *et al.*, (1990)
-antioxidant activity	van den Berg *et al.*, (1995); Liew *et al.*, (1995);
-no antioxidant activity	Chen *et al.*, (1997); Zhang and Chen, (1997)
Tumor Cells	
-prooxidant activity	Schønberg and Krokan, (1995)
-no oxidation effects	Wong *et al.*, (1997); Cunningham *et al.*, (1997)
Immune Cells	not yet studied

Table 1.4. Current Literature Regarding the Antioxidant Activity of CLA

As Zhang and Chen (1997) suggest, it is theoretically unlikely that CLA acts as an antioxidant as its free radical intermediate may not be stable and may further degrade. The production of furan fatty acids from CLA oxidation support this (Yurawecz *et al.*, 1995).

The effects of CLA on free radical production by immune cells have not been directly studied. *In vitro* work by Chew et al. (1997) and *in vivo* work by Wong et al. (1997) demonstrated that CLA enhanced or did not alter lymphocyte cytotoxicity and bactericidal activity, respectively. The exact mechanisms of these effects were not examined however. Therefore, research to date makes it difficult to draw any inferences regarding CLA's effects on the production of oxygen reactive species by immune cells.

One such compound which may be important in anti-tumor defense is nitric oxide (NO), a lytic compound produced from arginine via the nitric oxide synthesizing pathway. NO is produced by different immune cells including macrophages (MacMicking *et al.*, 1997), natural killer (NK) cells (Filep *et al.*, 1996) and cytotoxic T lymphocytes (Chinje and Stratford, 1997). Cells which lyse in response to NO may include viruses, bacteria, fungi, protozoa, helminths, and tumor cells (MacMicking *et al.*, 1997).

Several studies have examined the effect of dietary fat on macrophage production of oxygen reactive substances which might, in turn, alter immune function by enhancing cytotoxicity. Khair-El-Din *et al.* (1996) found that murine macrophages cultured with 3 μM DHA inhibited NO production. In contrast, Yaqoob and Calder (1995) showed that mice fed diets high in fish oil (20% w/w) had enhanced macrophage production of NO, superoxide and hydrogen peroxide compared to animals receiving low fat diets (2.5% w/w) or similar levels of hydrogenated coconut oil.

It is difficult to compare the results in the various studies due to *in vivo* versus *in vitro* work, dietary fat composition and other potentially confounding factors. Nonetheless, current CLA literature does suggest a few things. Ip *et al.* (1991) only saw the antioxidant effects of CLA on mammary tissue but not liver. Schønberg and Krokan (1995) found CLA to increase malondialdehyde (MDA) levels only in lung adenocarcinomas but not in glioblastoma cell lines. This suggests that if CLA acts as an antioxidant, its effects may be cell-specific. In addition, Ip *et al.* (1991) showed that CLA's antioxidant activity maximized at 0.25% (w/w) whereas its anticarcinogenic activity maximized at 1% (w/w). Schønberg and Krokan (1995) found that even when vitamin E was added *in vitro* to inhibit CLA-induced lipid peroxidation, cell growth did not fully return to normal levels. Both these studies suggest that CLA may act through mechanisms in addition to oxidative effects to inhibit tumorigenesis.

Generally, it would appear that fatty acids and dietary fat composition modulate production of oxygen reactive species such as nitric oxide. To clarify CLA's potential actions as an antioxidant, several factors need to be considered in future research. These include physiologically relevant *in vitro* models, dietary designs which mimic human lipid patterns for future *in vivo* studies and appropriate assays to measure peroxidation levels (Smith and Anderson, 1987; van den Berg *et al.*, 1995).

F) Changes in Membrane Composition

Lipids are important as a main component in cell membranes. Fluidity of this phospholipid bilayer is variable depending upon the PL, amount of cholesterol present, FA chain length and the degree of unsaturation (Innis and Clandinin, 1981; Field *et al.*, 1989; Garg *et al.*, 1990). The structure of the PL bilayer is demonstrated in ***Figure 1.3***.

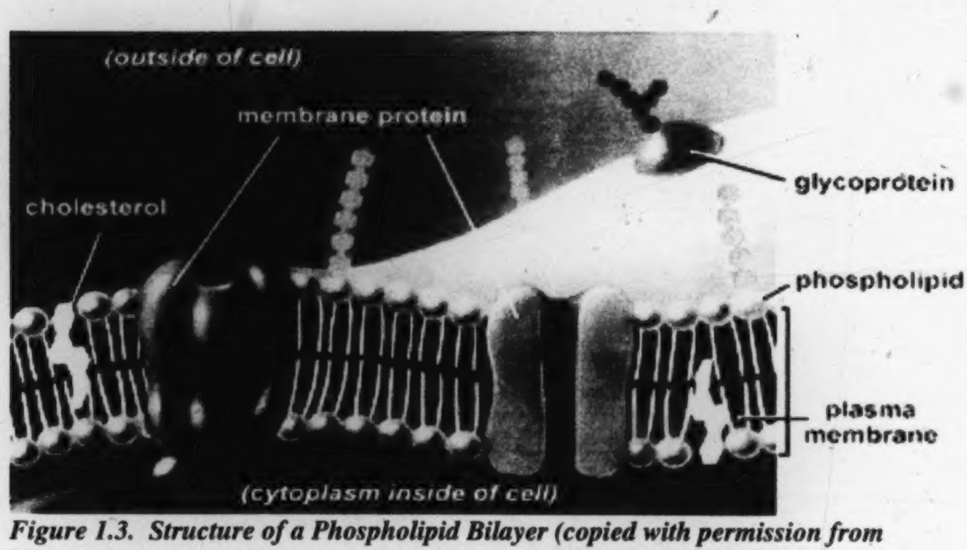

Figure 1.3. Structure of a Phospholipid Bilayer (copied with permission from http://info.bio.cmu.edu/courses/biochemmols/buildblocks/Gel.html)

Cell division has been shown to be associated with both membrane fluidity (Fischer, 1985) as well as an increased concentration of unsaturated fatty acids in the cell membrane (Belury *et al.*, 1991). In particular, LA exists at higher concentrations in the membranes of dividing cells (Cook *et al.*, 1993). It seems plausible that in a disease state such as cancer where rapid cell division is a concern, altering the dietary lipid composition might be a means of modifying cell proliferation and differentiation.

Changes in dietary lipid composition have been shown to result in concomitent changes in membrane composition in a variety of cell types including adipocytes (Field *et al.*, 1989), cardiac cells (Innis and Clandinin, 1981), hepatocytes (Garg *et al.*, 1988) and immune cells (Robinson and Field, in preparation). Changes in membrane composition may lead to changes in receptor function. Such changes have been found with adipocyte insulin binding, glucose transport and utilization (Field *et al.*, 1989), and ATPase-catalyzed adenosine triphosphate (ATP) / ^{32}P P$_i$ exchange (Innis and Clandinin, 1981). Cell permeability to various ions has been shown to be modifiable with dietary fatty acids (Devor and Frizzell, 1998). In addition, enzymes including Δ6 desaturase (Garg *et al.*, 1988) and phosphocholinetransferase (Hargreaves and Clandinin, 1987), glucose transporters (Sandra *et al.*, 1984) and second messenger pathways such as protein kinase

C (PKC; Chapkin *et al.*, 1997), phospholipase C-$\gamma 1$, ceramide production (Jiang *et al.*, 1996) and diacylglycerol (Marignani and Sebaldt, 1995) have been found to exhibit altered activity with changes in dietary fat. Thus, CLA may incorporate into membranes and effect receptor or enzyme function, cell permeability or second messenger pathways. These possibilities have not yet been explored.

Other cellular changes due to changes in membrane composition include antigen presentation by Class I and Class II major histocompatibility (MHC) products (Hughes *et al.*,1996) and the expression or function of cell surface proteins such as adhesion molecules as previously discussed. Finally, metabolic cooperation (i.e. intercellular communication and synchrony) between cells via changes at the gap junction may be altered with dietary lipid composition (Jiang *et al.*, 1997). Hii and colleagues (1995) demonstrated that some unsaturated fatty acids (ex. LA, oleic acid) inhibited metabolic cooperation between cells. Longer fatty acid carbon chain length has also been shown to decrease metabolic cooperation as does a *cis* geometric configuration (Welsch, 1987). A decrease in cell-to-cell communication might be expected in tumorigenesis as the tumor's growth and division are no longer controlled.

As the aforementioned studies indicate, there are numerous ways in which CLA, as a dietary fatty acid, could inhibit tumor development via membrane incorporation.

i) Incorporation into Various Tissues

CLA incorporates into the cell membranes of various tissues. Chin *et al.* (1994) examined the CLA concentration in liver, lung, skeletal muscle, and abdominal adipose and found that rats fed 5% (w/w) free LA had significantly higher CLA concentrations in these tissues than their germ-free counterparts ($p<0.02$) who were seemingly unable to synthesize CLA. CLA incorporation appears to increase as dietary levels of CLA increase. Ip *et al.* (1991) determined maximal incorporation in liver PL was achieved at CLA diet concentrations between 1 and 1.5% (w/w) in a rat model. CLA incorporation also increases with increasing dietary LA as demonstrated by Chin *et al.* (1994) who found that CLA was increasingly incorporated into liver, lung, skeletal muscle, and abdominal adipose PL with increasing dietary free (non-esterified) linoleic acid but not linoleic acid fed in a TG form. This suggests that the microbial flora convert free LA but not esterified LA into CLA which is subsequently incorporated into the PL. Similar results were found in humans by Herbel *et al.* (1998) when healthy humans were fed TG-esterified LA for six weeks with no resultant increase in plasma CLA levels. These studies suggest that free LA and TG-esterified LA may be metabolized differently.

CLA was found to incorporate into the neutral lipid (i.e. TG) and PL fractions of liver (Chin *et al.*, 1994) indicating a selectivity of incorporation of CLA into certain lipid classes. Chin *et al.* (1994) also determined that CLA incorporation (as a percent of total fatty acids) into the neutral lipid fraction of liver tissue was more predominant than in the PL fraction which suggests variation in the relative levels of incorporation in different lipid classes. Similar results were obtained by Ip *et al.* (1996) in the examination of mammary tissue. Therefore, it would seem that CLA is found predominantly in TG, the form in which most body fat is stored (Hunt and Groff, 1990).

CLA incorporation was not found to significantly alter the concentrations of other fatty acids. Notably, no significant alterations in CLA's parent compound, LA, were noted in rat mammary tissue (Ip *et al.*, 1996; Ip *et al.*, 1994C), rat colon (Liew *et al.*, 1995), and mouse forestomach (Ha *et al.*, 1990) with CLA incorporation. Similarly, other fatty acids such as AA and linolenic acid (LNA) in rat colon (Liew *et al.*, 1995), and oleic acid in mouse forestomach (Ha *et al.*,1990) were not changed by the incorporation of CLA.

However, the finding that CLA does not specifically displace other lipids in tissues and membranes is not universal. Cook *et al.* (1993) noted a decrease in the AA and LA content of fat pads in rats fed 0.5% (w/w) CLA diets compared to controls. Belury and Kempa-Steczko (1997) fed rats diets supplemented with 0, 0.5, 1.0, or 1.5% (w/w) CLA and demonstrated that CLA was incorporated into hepatic neutral lipids and PL at the expense of LA. In neutral lipids, oleic acid and AA decreased in CLA-fed animals. LA was found to decrease in liver PL. The reduction in AA and LA suggest that CLA may interfere in their metabolism. This group also found that CLA was desaturated to an 18:3 product similarly to the desaturation of LA. This suggests that the Δ6 desaturase does not preferentially use LA or CLA however competition would still exist in that both fatty acids can be metabolized by this enzyme. An indirect demonstration of displacement of other fatty acid classes with CLA supplementation was noted by Ip *et al.* (1996) who found that feeding rats a CLA-free, 20% (w/w) fat diet led to a significant increase in incorporation of LA in the PL of mammary gland.

Selective displacement of other fatty acids (i.e. most notably LA and AA) might occur in some tissues but not others. This is plausible when one considers that CLA's effects are also tissue specific. Changes in the membrane fatty acid profile and competition between CLA and other fatty acids for various metabolic pathways (i.e. eicosanoid synthesis) may prove to be important anti-tumorigenic mechanisms and require further clarification.

In addition to a preferential incorporation into certain lipid classes, there may be preferential isomeric incorporation, specifically the *c*9,*t*11 form which has been shown to occur in mammary epithelial cells (Ip *et al.*, 1994C), forestomach (Ha *et al.*, 1990), mammary tumor PL and liver PL (Ip *et al.*,1991) suggesting that the *c*9,*t*11 isomer is the biologically active form as discussed previously.

As suggested by Ip *et al.* (1994B), analyses of CLA concentrations in the various lipid fractions can be difficult, particularly for the mammary epithelial PL (a fraction of interest in studying mammary adenocarcinomas) which comprises only about 1% (w/w) of the total extractable fat in the mammary gland compared to the more abundant TG fraction. Therefore it will be imperative to develop precise ways to measure such small lipid fractions. As proposed by Ip and colleagues, (1994C), CLA in mammary epithelial cell PL may prove to be a relevant indicator of cancer resistance. Without the means to accurately measure these smaller lipid fractions, it will be difficult to study such possibilities.

ii) Incorporation into Tumor Cells

Ip *et al.* (1991) examined the incorporation of CLA into mammary tumor PL and found similar trends to those described above. The *c*9,*t*11 isomer was incorporated in proportion to dietary intake and at a greater magnitude than that for liver PL (20-fold vs. 6-fold increase). Dietary CLA up to levels of 1.5% (w/w) were studied, however, unlike liver PL which reached maximal incorporation at between 1% and 1.5%, the mammary tumor showed no evidence of reaching maximal CLA incorporation at a dietary concentration of 1.5% (w/w).

iii) Incorporation into Immune Cells

The incorporation of CLA into immune cell membranes and the specific PL classes has not been studied but would provide useful information since dietary CLA has been shown to alter immune function. In addition, determining the specific PL classes which incorporate CLA would prove helpful in elucidating the second messenger pathways involved. Many immune functions depend on the concentration of lipids in the membrane and may be modulated by dietary lipid. This will be discussed shortly.

G) Eicosanoid Production

Lipid metabolism in the body results in the production of eicosanoids as outlined in ***Figure 1.4***.

LINOLEIC ACID / LINOLENIC ACID (IN MEMBRANE PL)
↓ (Phospholipase A_2)
ARACHIDONIC ACID / EICOSAPENTAENOIC ACID

↓ (Cyclooxygenase Pathway) ↓ (Lipoxygenase Pathway)
↓ ↓
↙ ↘ ↙ ↘

PROSTAGLANDINS THROMBOXANES HETEs HODEs
↓
LEUKOTRIENES

Figure 1.4. Biosynthetic Pathways for Eicosanoid Production

Both ω-6 (ex. AA) and ω-3 (ex. EPA) fatty acids serve as precursors for eicosanoids which consist of prostaglandins (PG), leukotrienes (LT), thromboxanes (TX) and hydroxy eicosatetraenoic acids (HETE). The cyclooxygenase and lipoxygenase pathways that produce eicosanoids compete for these precursors. PG affect such functions as platelet aggregation, diuresis, decreased blood pressure, effects on the immune, nervous and digestive systems, and the stimulation of smooth muscle contraction (Hunt and Groff, 1990). Certain TX exert antagonistic effects to those of the PG including platelet aggregation and vasodilation of blood vessels. LT act to contract smooth muscle in the respiratory, digestive and vascular systems.

Eicosanoids, especially PGE_2 and LTB_4, have been implicated as important immunomodulators (Brouard, 1993). PGE_2 is produced by most mammary tumors (Zhao *et al.*, 1996) and is often associated with enhanced tumorigenesis and suppressed T-cell function. PGE_2 has been shown to inhibit NK cell activity (Brunda, 1980), lymphocyte proliferation (Goodwin, 1977), cytotoxicity (Leung, 1980) and other lymphocyte functions (Lewis, 1983). LTB_4 enhances NK cell activity (Rola-Pleszczinski, 1983) and proliferation of IL-2 dependent T cells (Atluru, 1986) but inhibits general human T cell function (Payan, 1983). In addition, eicosanoids (specifically 12-HETE and 13-HODE) have been shown to enhance adhesion of tumor cells to endothelial cells via up-regulated integrin expression (Tang *et al.*, 1994). The importance of adhesive changes was discussed previously.

Dietary fat has been shown to alter eicosanoid production. For example, fish oil (DHA and EPA) alters eicosanoid production by competing with AA for the enzymes in the cyclooxygenase and lipoxygenase pathways. Whereas AA is metabolized to PG of the "2" series, EPA will compete for the same enzymes to produce PG of the "3" series. In many cases, these two series will have different and even opposing physiological effects (eg. inflammatory nature of PGE_2 versus anti-inflammatory nature of PGE_3). CLA may be immunomodulatory as a competitive inhibitor or by acting as a substrate for the enzymes in these pathways to produce a completely different series of eicosanoids with unique immunomodulatory properties. Alterations in the balance of different eicosanoids might result in altered thrombotic abilities, immune function, or digestive capabilities. These possibilities have yet to be studied, although preliminary work (Cunningham *et al.*, 1997) incubating the MCF-7 cell line with CLA and nordihydroguaiaretic acid (NDGA; a lipoxygenase inhibitor) suggests that CLA exerts its inhibitory effects through the lipoxygenase pathway. Likewise, Liu and Belury (1997) found that incubating CLA with murine keratinocytes resulted in less PGE release than incubating LA or AA. These studies suggest that CLA may exert its effects via eicosanoids. However, it is not yet elucidated as to which specific pathways and enzymes may be affected.

Ip *et al.* (1996) suggested that the small change in CLA in mammary gland PL argued against the significance of a membrane effect. Further, Belury *et al.* (1996) suggested that the greater incorporation of CLA into neutral lipids compared to PL could indicate that CLA's anticarcinogenic effects are more dependent on modulation of fat metabolism as opposed to membrane effects. Although quantitative measures of CLA incorporation are important and may hint at potential physiological mechanisms, it would seem dangerous to exclude the importance of incorporation into the PL based purely on these quantitative measures. These groups were looking for membrane incorporation of CLA but did not examine the long chain desaturated products that might be expected if CLA were incorporated into membrane. Such elongated products or the displacement of other elongated products might provide insight into the importance of PL incorporation of CLA.

The modulatory effects by CLA on eicosanoid production require further direct investigation. The effects of CLA on the various enzymes as well as its effects on eicosanoid production in different cells (i.e. immune cells, tumor cells) must be examined to allow more definitive conclusions to be drawn.

As previously discussed, the effect of CLA incorporation into cell membranes on the levels of other fatty acids is not clear. These previous studies were performed on animals that were receiving diets unlike human dietary fat patterns in both fat level and composition. Future studies to characterize the effects of dietary CLA on other fatty acid levels and eicosanoid production should utilize diets which are more representative of human dietary fat composition. This will provide a more physiologically relevant model to investigate anti-tumorigenic mechanisms.

H) Actions on Anti-tumor Immune Defenses

As proposed by the immune surveillance hypothesis (Burnet, 1970), the immune system is actively involved in detecting and eradicating neoplastic cells. There are several immune cell populations believed to be involved in responding to a developing tumor.

Natural killer (NK) cells are large granular lymphocytes which have been shown to serve as a rapidly-acting first line of defense against tumors, particularly against metastasis (Hanna, 1985). NK cells demonstrate non-MHC restricted cytotoxicity and exert their effects when MHC Class I molecule expression is reduced (Timonen, 1997). Lysis by this immune population is believed to be carried out by perforin, granzymes, and NO (Filep *et al.*, 1996). NK activity has been found to be regulated by the products of AA metabolism. More specifically, PGE_2 and LTB_4 have been implicated as important modulators (Vaillier *et al.*, 1992) leading to a reduction in NK activity. NK cells are able to metabolize AA and to synthesize eicosanoids from both the lipoxygenase and cyclooxygenase pathways (Cifone *et al.*, 1993).

Macrophages have also been found to be involved in tumor defense through secretion of lytic enzymes and cytotoxic products such as tumor necrosis factor-alpha (TNF-α) (Kuby, 1994). This population of cells have been shown to exist in higher concentrations around tumors and to destroy neoplastic cells *in vitro* (Herberman, 1983). Tumor cells do not appear to develop resistance to macrophage cytotoxicity. Macrophage killing ability can be altered by diet- particularly dietary lipid (Black and Kinsella, 1993; Somers *et al.*, 1989). This change in cytotoxicity has been suggested to be due to altered eicosanoid production.

In addition, cytotoxic T-lymphocytes (CTL) are important in tumor immunology. Various tumors have been reported to secrete tumor-associated antigens (TAA) which may be aberrant forms or over-expressed forms of normally expressed intracellular antigens, tissue-specific differentiation antigens, or antigens of viral origin. Examples include mucin-like carcinoma-associated antigen (MCA), tumor polypeptide antigen (TPA), and carcinoembryonic antigen (CEA) (Pectasides *et al.*, 1996). TAA such as these are presented by MHC Class I molecules for recognition by CTL which then lyse the tumor cell.

TAA can also result in the production of tumor-specific antibodies (TSA) which initiate tumor cell lysis via a variety of mechanisms including complement activation and antibody-dependent cell-mediated cytotoxicity (Kuby, 1994). Various pathways involved in anti-tumor immune defenses can be found in ***Appendix A***.

There are several ways in which tumors attempt to evade immune detection. Often a tumor will down-regulate expression of MHC Class I molecules so that TAA are less effectively presented to CTL for immune detection. Although this may effectively reduce CTL tumor lysis, it is this very signal which initiates NK cytotoxicity. Tumors often lack expression of MHC Class II (Panelli *et al.*, 1996) and other accessory molecules (Restucci *et al.*, 1997; Nilsson *et al.*, 1996) so that, even if TAA are presented, the CTL lack the help they require to elicit an effective immune response. Thirdly, tumors may secrete various cytokines such as transforming growth factor β (TGF-β) which have been shown to suppress cellular immunity (Yotsukura *et al.*, 1997). As well, since TAA are at low serological levels, the suppressor cell population develops which reduces immune efficacy by decreasing the CTL response. Various pathways involved in evasion of the immune system by the tumor are also outlined in ***Appendix A***.

i) CLA and Immune Function

The effects of CLA on immune function have been examined by several groups. Chew *et al.* (1997) examined porcine lymphocyte and murine macrophage function *in vitro* and found that physiological levels of CLA stimulated mitogen-induced lymphocyte blastogenesis, lymphocyte cytotoxic activity and macrophage killing ability but decreased IL-2 production.

A study by Cook *et al.* (1993) revealed that rats fed 0.5% (w/w) CLA for four weeks demonstrated enhanced phytohemagglutinin (PHA) response and macrophage phagocytosis following lipopolysaccharide (LPS) injection compared to control rats. In addition, CLA was not found to affect antibody levels to sheep red blood cells in chicks or bovine serum antibody in rats. CLA was found to prevent the catabolic effects normally seen with immune stimulation. They concluded, however, that CLA did not suppress immune function. Further evidence for immune enhancing effects were reported by Miller *et al.* (1994) who determined that feeding a 0.5% CLA diet to mice resulted in enhanced spleen lymphocyte blastogenesis in response to endotoxin injection. Finally, Wong and colleagues (1997) found that feeding 0.3-0.9% CLA to mice for 3 or 6 weeks resulted in enhanced PHA-induced lymphocyte proliferation and IL-2 production with no effects on lymphocyte cytotoxicity. Despite these effects, CLA was not found to be effective against the development of an aggressive mammary tumor.

Beyond these studies, the potential immunomodulatory effects of CLA in both the normal state and during cancer are largely unstudied making this is a particularly exciting field for future research.

ii) Dietary Fat and Immune Function

Many studies have examined the effects of other fatty acids on immune function however the results are sometimes conflicting. This is not surprising when one considers the complexity of such a system. Many factors may regulate a single immune component. For example, NK cells are affected by several cytokines including IL-1, IL-2, IL-12, and interferon gamma (IFN-γ). As well, a single immune component may secrete many immunomodulating substances. For example, macrophages secrete IL-1, IL-6, IL-8, IL-12, and TNF-α (Kuby, 1994). There are a vast number of interactions between the various components so that, depending on the experimental conditions, different and apparently conflicting results might be obtained. *In vitro* work examining the effects of a single immune factor is important however in attempting to elucidate potential cellular mechanisms.

Dietary fat does affect immune function. Many studies have examined the effects of dietary fish oil. For example, Robinson and Field (1998) found that fish oil-enriched diets led to increased NK cell activity. Brouard and Pascaud (1993) and Field and Goruk (submitted) reported decreased unstimulated lymphocyte proliferation of rat splenic lymphocytes with Con A and PMA + Iono compared to rats consuming a control diet. Somers and Erickson (1994) found that peritoneal macrophage cultures from mice fed fish oil-supplemented diets exhibited higher TNF-α activity after LPS stimulation than did the macrophages from mice given a diet high in ω-6 fatty acids. They proposed that ω-3 fatty acids resulted in a loss of clearance of TNF-α rather than an increase in bioactivity or production. In contrast, a study in humans demonstrated decreased TNF-α levels when ω-3 fatty acids were given in the diet (Meydani, 1991).

Somers *et al.* (1989) found that peritoneal macrophages from mice given a diet containing 10% (w/w) menhaden fish oil exhibited hyporesponsivity to IFN-γ compared to diets containing similar levels of safflower oil or hydrogenated coconut oil. In contrast, they found that macrophages from fish oil-fed mice were highly cytolytic compared to the safflower oil-fed group when activated with LPS.

It appears that there is more than one mechanism whereby dietary fat may alter immune function and it may affect different components of the immune system differently. Dietary fat has been shown to influence the expression of genes involved in effective immune responses (Huang *et al.*,1992; Khair-El-Din *et al.*, 1996) such as major histocompatibility class II antigens. Changes in immune cell populations have also been proposed (Huang *et al.*, 1992). However, the most popular mechanistic explanation is altered eicosanoid production as previously discussed. The fatty acid composition of immune cells (ex. macrophages, splenocytes) has been reported to be dependent on the lipid composition of the diet (Somers *et al.*, 1989; Robinson and Field, submitted) supporting this as a potential mechanism.

IV. Directions for Future Research

Based on the literature to date, the mechanism for a beneficial effect of CLA remains to be elucidated. However, certain possibilities appear to be more viable as venues for future research. The effects of CLA on immune function would be valuable to pursue. Research to date indicates immunomodulatory effects of CLA (i.e. enhanced immune proliferation and lymphocyte cytotoxicity), however greater investigation, particularly regarding effects on anti-tumor immune defenses, might elucidate an anticarcinogenic mechanism.

Another basic issue which has not been well addressed involves the metabolism and incorporation of CLA in the body. It has not yet been clearly determined whether dietary CLA results in alterations in specific fatty acid levels in cell membranes and, if so, whether such membrane composition changes are specific to certain tissues. Examination of incorporation of CLA and its metabolites into the mammary gland, tumor, and immune cells would be relevant as these might also result in functional changes in these tissues. It would also be worthwhile to study the effects of CLA on eicosanoid production in the mammary gland, tumor, and in various immune cells. Immunomodulation via changes in eicosanoid production appears to be a reasonable mechanism whereby CLA might inhibit mammary tumorigenesis and would therefore be a worthwhile area to pursue. This might potentially lead to greater knowledge regarding tumor initiation and development. Thirdly, it would be beneficial to examine the effects of CLA on expression and/or function of various accessory molecules, including adhesion molecules. These molecules have been demonstrated to be modifiable by other fatty acids and are important in the process of metastasis, a process which CLA has been shown to reduce.

Perhaps one of the most important considerations for future CLA research is careful dietary design. Previous research has not often considered the effects of dietary fat level and composition on the efficacy of CLA nor attempted to formulate diets to simulate human dietary lipid patterns. These considerations are imperative if the anti-tumorigenic effects of CLA seen in animal models are to be extrapolated to recommendations for the human population.

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Chapter Two- Study Rationale

I. Introduction

CLA has been shown to be anticarcinogenic in several animal models (Ha *et al.*, 1987; Ha *et al.*, 1990; Ip *et al.*, 1991; Liew *et al.*, 1995) . As discussed previously, several potential mechanisms have been hypothesized however no mechanisms of action have yet been elucidated. Lipids are known to alter immune function (Huang *et al.*, 1992; Black and Kinsella, 1993; Erickson and Hubbard, 1996). For example, PUFA such as LA have often been shown to be immunosuppressive. Therefore, as a lipid, CLA may be immunomodulatory which may be an antitumorigenic mechanism (Erickson and Hubbard, 1994). Evidence for such a mechanism exists both *in vitro* (Chew *et al.*,1997) and *in vivo* (Miller *et al.*,1994; Wong *et al.*, 1997), however, the effects of dietary fat level and composition on the immunomodulatory effects of CLA were not considered in these studies.

Dietary CLA studies in animal models have largely been conducted by adding CLA to standard laboratory rodent diets which differ from human diets in both fat level and composition. Studies suggest that fatty acid composition (Ip *et al,* 1985; Ip, 1997) and level (Welsch *et al,* 1990) can modulate tumor development. The anticarcinogenic effects of CLA may be similarly modulated by these factors. Therefore, dietary fat level and composition should be considered in designing appropriate dietary treatments.

In addition to potential effects on immune function, CLA appears to act via other mechanisms (Visonneau *et al.*, 1997). The effects of CLA on adhesion have not yet been studied, however research examining other fatty acids suggests that this may be an anti-metastatic mechanism (Lu *et al.*, 1995; Johanning and Lin, 1995; Collie-Duguid and Wahle, 1996).

Despite the differential effects on adhesion which have been found, it is clear from the literature that ω-3 fatty acids reduce tumor metastasis and affect cell adhesion, function and expression. Conversely, it has been found that LA also enhances tumor metastasis and tumor cell adhesion. In light of the opposing effects of LA and CLA and the similar effects of EPA/DHA and CLA on tumor growth, development and metastasis, it seems logical that CLA as a dietary fat may affect tumor metastasis by modulating cell adhesion.

II. Hypotheses and Objectives

It is hypothesized that supplementing CLA in a diet containing similar lipid level and composition to what humans consume will result in physiological effects, some of which may enhance anti-tumor defenses.

Study One

Experiments were designed in a rat model to determine the effects of feeding CLA in a diet with a fat level and composition consistent with that consumed by humans on:

- anti-cancer immune defenses
- food intake
- weight gain and body composition
- CLA incorporation into adipose TG and immune cell membranes

It is hypothesized that:

a) CLA will enhance anti-cancer immune defenses when fed in diets that are representative of current recommended human dietary patterns.

b) P/S ratio will modulate anti-cancer immune defenses when fed in diets that are representative of current recommended human dietary patterns. Low P/S diets will enhance immune function compared to high P/S diets.

c) The efficacy of CLA will differ with P/S ratio. Low P/S will augment the effects of CLA on immune function compared to high P/S diets.

d) CLA supplementation will reduce food intake.

e) CLA supplementation will not change body weight gain but will modulate body composition with a reduction in body lipid and an increase in body protein.

f) Dietary lipid composition will result in different total and isomeric CLA incorporation into adipose tissue and immune cell membranes. Low P/S diets will result in greater incorporation of CLA than will high P/S diets.

Study Two

Experiments were designed in a cell culture model to determine the effects of incubating various fatty acids, including CLA, on:

- tumor cell proliferation
- tumor cell membrane fatty acid composition
- adhesion of tumor cells to various basement membrane substrates
- expression of adhesion molecules on the tumor cell surface.

It is hypothesized that:

a) The addition of CLA to tumor cell cultures will reduce tumor cell proliferation relative to controls.

b) The addition of CLA to tumor cell cultures will result in changes in tumor cell membrane composition.

c) CLA will reduce adhesion of tumor cells to various basement membrane components.

d) CLA will reduce expression of adhesion molecules (specifically β_1 and β_4 integrins) on the tumor cell surface.

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Chapter Three- Conjugated Linoleic Acid and Immune Function in Healthy Rats

I. Introduction

Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of linoleic acid (LA). Although CLA is a relatively minor dietary constituent, it has recently received attention due to potent anticarcinogenic effects which have been demonstrated in animal models of skin (Pariza and Hargraves, 1985), forestomach (Ha *et al.*, 1990), colon (Liew *et al.*, 1995) and mammary cancer (Ip *et al.*, 1991). Inhibitory effects of CLA have also been noted in several tumor cell lines (Seaman *et al.*, 1992; Shultz *et al.*, 1992, 1993; Schønberg and Krokan, 1995; Yoon *et al.*, 1997; Cornell *et al.*, 1997).

Although CLA's anticarcinogenic effects are well established, potential mechanisms of action in reducing tumorigenesis have yet to be elucidated. As proposed by the immune surveillance theory (Burnet, 1970) the immune system is actively involved in detecting and eradicating neoplastic cells in the body. For example, cytotoxic T lymphocytes (CTL), natural killer cells, macrophages, and tumor antigen-specific antibodies are all involved in the immune response to tumors (Kuby, 1994). Lipids are known to alter immune function (Huang *et al.*, 1992; Black and Kinsella, 1993; Erickson and Hubbard, 1996). Therefore, as a lipid, CLA may be immunomodulatory which may be an antitumorigenic mechanism (Erickson and Hubbard, 1994).

Some work has been done examining the effects of dietary CLA on immune function (Miller *et al.*, 1994; Wong *et al.*, 1997; Sugano *et al.*, 1998; Hayek *et al.*, 1999). None of these studies considered the effects of dietary fat level and composition when examining the immunomodulatory effects of CLA. Dietary CLA studies in animal models have largely been conducted within the context of laboratory rodent diets which differ from human diets in both fat level and composition. Standard laboratory chow is lower in fat (approximately 5% (w/w) or 10% of energy) and contains much more polyunsaturated fat (P/S ratio ≥4) than human diets. The current human dietary lipid recommendation by Health and Welfare Canada (1990) is 30% of energy- much higher levels than those seen in laboratory rodent diets. The current human lipid composition recommendation (1990) is a P/S=1.0- a much lower ratio compared to those seen in laboratory chow diets. It is difficult to extrapolate the anticarcinogenic effects of CLA in animals models to potential benefits for human tumor management and prevention when differing dietary composition may confound the results. Dietary lipid differences may also be of particular significance in light of the possibility of competition between CLA and other fatty acids such as LA.. Such competition may modulate the physiological effects of CLA and should, therefore, be considered in designing appropriate dietary treatments.

The purpose of the following experiment was to examine the effects of feeding CLA on anti-cancer immune defenses and CLA incorporation into adipose tissue and immune cell membranes in animals fed diets with a fat level and composition consistent with that in the human diet.

II. Materials and Methods

A) Experimental Design

Experiments were reviewed by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care. Forty weanling male and female Fischer 344 rats were randomized to one of four diet treatments (***Figure 3.1***). Weanling rats, as opposed to adult animals, were chosen to allow comparison with several previous studies which examined younger animals. Thompson *et al.* (1997) also demonstrated that CLA had effects on the development of the mammary gland which might reduce mammary susceptibility to mammary tumorigenesis. The experimental time frame of the current study covered this time of mammary gland development which occurs from weaning to approximately 50 days (Russo *et al.*, 1982).

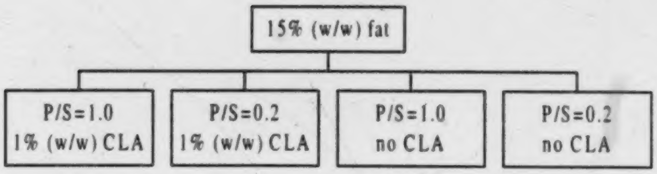


Figure 3.1. Diet Design. *The dietary fat level and compositions of the four diets used in the study.*

All diet treatments were nutritionally complete, semi-purified and contained 15% (w/w) or approximately 30% of total energy as lipid. Two diets were supplemented with 1% (w/w) CLA (in FFA form) and the remaining two were not. Within these two groups (CLA-supplemented and non-supplemented), diets had a fat composition providing a P/S ratio of either 1.0 or 0.2. These compositions were achieved through a combination of three fat sources: safflower oil, linseed oil, and stearine. The CLA which was supplemented was produced in our laboratory as described elsewhere (Ma *et al.*, in press B). Diets met requirements for LA (18:2n-6) and LNA (18:3n-3). See ***Appendix B*** for a complete summary of diet composition.

Animals were housed individually in a temperature and light-controlled room (12 hour light/dark cycle), had free access to water and were fed *ad libitum* one of the four diets described above. During the three week feeding period, body weight and food intake were recorded on alternate days. Rats were then terminated by CO_2 asphyxiation. Spleens were removed under sterile conditions and placed in chilled sterile petri dishes containing Krebs-Ringer Hepes buffer (KRH) with 5% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). Epidydymal fat pads were also removed from males and frozen at -70°C for later lipid analysis. Carcasses were stored at -20°C for later analysis of body composition.

B) Lymphocyte Isolation

Under sterile conditions, spleens were pressed through a nylon mesh (100 µm) which was then washed several times with KRH + 5% (w/v) BSA to maximize splenocyte recovery. Samples were then centrifuged (Beckman J2-HC, Beckman

Instruments, Palo Alto, CA) for 10 minutes at 1250 rpm to pellet cells. The supernatant was removed and cells were resuspended in 1.5 mL of lysis buffer (see ***Appendix C*** for composition) for three minutes to lyse red blood cells. After exactly 3 minutes, 25 mL of KRH + 5% (w/v) BSA was added to dilute the lysis buffer and cells were again spun at 1250 rpm for 10 minutes. Subsequently, the supernatant was removed and cells were resuspended in 5 mL of complete culture media (CCM; RPMI 1640; Gibco BRL, Grand Island, NY) with 4% fetal calf serum (FCS; Gibco BRL, Grand Island, NY) and 2.5 µmol/L 2-mercaptoethanol (ICN Biochemicals, Cleveland, OH). Viability was determined by trypan blue exclusion (Sigma Chemical Co., St. Louis, MO).

C) Natural Killer Cytotoxicity

Cytotoxicity was determined via ^{51}Cr release according to the method described by Field *et al.* (1991). YAC-1 cells, an NK-sensitive cell line (a gift from Dr. Rabinovitch, Department of Medicine, University of Alberta, Edmonton, AB), were spun for 10 minutes at 1250 rpm, resuspended in 1 mL 100% FCS and counted for viability using trypan blue exclusion. ^{51}Cr sodium chromate (250 µCi; Amersham, Ontario, Canada) was added to 6.0 x 10^6 YAC-1 cells (enough for 6 rats) and enough 100% FCS was added to bring the volume to 1 mL. The YAC-1 were allowed to incubate at 37°C in a shaking water bath for 1 hour 20 minutes to allow uptake of radioactivity. Cells were gently washed of extracellular radioactivity by spinning the cells for 10 minutes at 1250 rpm, removing the supernatant and resuspending in 5 mL CCM with 10% FCS (v/v). This washing was repeated 3 times. In the final wash, cells were resuspended in 0.5 mL CCM with 20% FCS (v/v) and counted again for viability by trypan blue exclusion. Cells were then diluted to 0.2 x 10^6 cells/mL in CCM (20% FCS (v/v)). The cell suspension (50 µL) was added to 96 well V-bottom plates (Costar, Cambridge, MA). Immune cells, which had been isolated from spleen as described above, were prepared at a concentration of 10.0 x 10^6/mL for the 100:1 effector:target ratio. This was subsequently diluted to create ratios of 50:1, 25:1, 12.5:1, 5:1 and 2:1 for each rat. These solutions were added in triplicate (100 µL) to the radioactive YAC-1 cells. In addition, spontaneous lysis was determined by incubating YAC-1 cells with 100 μL of CCM and maximum lysis was determined by detergent lysis with 100 μL of CCM with 10% FCS (v/v) and 4% Triton-X (v/v) (BDH Chemicals, Toronto, ON). Plates were briefly spun until 1000 rpm was reached at which time the centrifuge was braked. Plates were then allowed to incubate for four hours at 37°C with 5% CO_2 to allow NK lysis of YAC-1 cells. Following incubation, plates were spun for 10 minutes at 1250 rpm. A 75 μL aliquot of the supernatant was counted in a gamma counter (Beckman 8000; Beckman Instruments, Palo Alto, CA). The percentage of specific lysis was determined using the formula:

% specific lysis= 100 x (experimental lysis (cpm)-spontaneous lysis (cpm)) /(maximum release (cpm)- spontaneous release (cpm))

D) Splenocyte Phenotyping

Splenocyte subpopulations were determined by immunofluorescence using antibodies derived from hybridomas which secrete mouse monoclonal antibodies directed against rat lymphocyte surface proteins. The different antibodies used and the lymphocyte populations recognized by each are summarized in the table below (***Table 3.1***). Immunofluorescent labels were fluorescent markers conjugated to goat anti-mouse IgG antibodies. The phenotypic assays utilized both single and double labelling with antibodies. Non-sterile 96 well V-bottom plates (Fisher, Edmonton, AB) were preconditioned with phosphate buffer solution (PBS) + 4% (v/v) FCS at room temperature for at least twenty minutes. Splenocytes (0.2-0.5 x 10^6) were added to each well and the first antibodies were added and incubated for at least twenty minutes at 4°C. Cells were then washed by pelleting the cells via centrifugation at 1250 rpm for three minutes, removing the supernatant, vortexing to break the cell pellet and adding 200 μL PBS + 4% FCS (v/v). This was repeated three times. The secondary immunofluorescent antibody label, fluorescein isothiocyanate (FITC) , was added and incubated for at least 20 minutes at 4°C. Cells were then washed three times as described previously. For double labelling, the same sequence was followed again with phycoerythrin (PE; CALTAG Laboratories, Burlingame, CA) serving as the secondary immunofluorescent label. Cells in each well were then fixed with 200 μL PBS + 1% (w/v) paraformaldehyde (Anachemia Science, Montreal PQ), acquired by flow cytometry on a FACScan (Becton Dickinson, Sunnyvale, CA) and analyzed (CellQuest, Becton Dickinson, Sunnyvale, CA). Negative controls were used to determine the amount of non-specific binding of the immunofluorescent labels to the cells (i.e. goat anti-mouse IgG binding to rat splenocytes) and were used to correct for background fluorescence.

Splenocyte phenotyping was carried out for freshly isolated cells as well as for splenocytes that had been stimulated with Concanavalin A (Con A) and cultured for 42 hours.

Cell Surface Marker	***Lymphocyte Population Recognized***	***Supplied by***
OX19	all T cells	Dr. Rabinovitch, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada
OX8	suppressor and cytotoxic T-cells (CD8+)	
w3/25	T-helper cells (CD4+)	
OX12	B-cells	
OX42	macrophages	
3.2.3	Natural Killer cells	Cedarlane Laboratories, Ontario, Canada
OX26	transferrin receptor	
OX39	IL-2 receptor	

Table 3.1 Monoclonal antibodies utilized in phenotyping.

E) Splenocyte Proliferation

Splenocytes were seeded into 96 well, round bottom, tissue culture-treated plates (200 μL of 1.25 x 10^6 cells/mL; Corning, New York, NY). Con A (20 uL of 5μg/mL) or phorbol myristate acetate (PMA; 10 μL of 40 ng/mL) and ionomycin (Iono; 10 μL of 0.5 nmol/mL) were added in triplicate or quadruplicate. Addition of CCM with 4% FCS (v/v) and 2.5 μmol/L 2-mercaptoethanol allowed determination of unstimulated proliferation. Plates were then covered and incubated at 37ºC with 5% CO_2. Cells were pulsed with 0.5 μCi ^{3}H-thymidine (Amersham, Ontario, Canada) at 24, 48 and 72 hours followed by an additional 18 hour incubation to allow thymidine incorporation. At 42, 66, and 90 hours, cells were harvested onto glass fiber filter mats (Skatron, Suffolk, UK) with a multiwell harvester (Skatron, Lier, Norway), transferred to scintillation vials containing Ecolite® and counted on a beta counter (Beckman 5000, Beckman Instruments, Palo Alto, CA). The total dpm for each well were used to determine thymidine incorporation due to proliferative response.

F) Interleukin-2 Production

Six to eight millilitres of splenocytes in CCM with 4% FCS (v/v) and 2.5 μmol/L 2-mercaptoethanol were prepared at a concentration of 2.5 x 10^6 cells/mL in sterile plastic 10 mL tubes. Con A was added in the same concentration as for the proliferation assay. Unstimulated cytokine production was determined by adding the same volume of CCM. Tubes were covered and incubated at 37ºC with 5% CO_2 for 48 hours. Cells were then pelleted by spinning at 1250 rpm for 10 minutes. The supernatant was transferred to sterile plastic 10 mL tubes and frozen at -70ºC for later cytokine and nitric oxide analysis. Stimulated cells were removed and counted for viability using trypan blue exclusion for a stimulated phenotyping assay as described earlier. Any cells not used in phenotyping were frozen at -70°C for later phospholipid analysis.

Supernatants were later thawed. Concentrations of IL-2 were determined via a colorimetric ELISA assay. All antibodies and standards were purchased from Pharmingen, San Diego, CA. Purified anti-cytokine capture antibody was diluted to 2 μg/mL in Binding Solution (see ***Appendix C*** for composition of all solutions in this protocol) and added (50 μL) to each well of Immulon® 2 HB high binding flat bottom microtiter plates (Dynex Technologies Inc., Chantilly, VA). Plates were sealed, incubated overnight at 4°C, and washed 4 times with PBS/Tween. Each wash involved adding 200 μL of PBS/Tween, gently shaking for 1 minute, inverting and blotting the plate on an absorbent surface. Non-specific binding was blocked by adding 200 µL of Blocking Buffer/well, sealing the plates and incubating at room temperature for 30 minutes. Plates were then washed 3 times with PBS/Tween as described previously. Standards (see ***Appendix C*** for concentrations used) and samples were then added in triplicate to wells. Standards were diluted in Blocking Buffer/Tween and 100 µL was added to each well. Stimulated samples were added (50 µL) and diluted with 50 µL of Blocking Buffer/Tween whereas 100 µL of unstimulated samples were used so that all wells contained equal volumes. Plates were then sealed, incubated for 3 hours at room temperature and washed 4 times with PBS/Tween.

Biotinylated anti-cytokine detecting antibody was diluted to 2.0 µg/mL in Blocking Buffer/Tween and 100 µL was added to each well. Plates were sealed, incubated at room temperature for 1 hour and washed 6 times with PBS/Tween. Avidin-horseradish peroxidase was diluted to 0.5 µL/mL in Blocking Buffer/Tween and 100 µL/well was added, plates were sealed, incubated at room temperature for 30 minutes, and washed 8 times with PBS/Tween. ABTS Substrate Solution was thawed 20 minutes prior to use. 10 µL of 30% H_2O_2/ 11 mL of substrate was added and vortexed. 100 µL of this solution was added to each well. Plates were incubated at room temperature for 30 minutes and the optical density was read at 405 nm on a plate reader (Model EL309; Bio-tek Instruments Inc., Burlington, VT).

G) Interferon Gamma Production

Supernatants were prepared and frozen at -70°C as described for IL-2. Concentrations of IFN-γ were determined via a colorimetric ELISA assay. All antibodies and standards were purchased from Genzyme Diagnostics, Cambridge, MA. Purified anti-cytokine capture antibody was diluted to 2 µg/mL in Coating Buffer (see ***Appendix C*** for composition of all solutions in this protocol) and added (100 µL) to each well of Immulon® 2 HB high binding flat bottom microtiter plates (Dynex Technologies Inc., Chantilly, VA). Plates were sealed, incubated overnight at 4°C, and washed 3 times with wash buffer. Each wash involved adding 200 µL of wash buffer, gently shaking for 1 minute, inverting and blotting the plate on an absorbent surface.

Non-specific binding was blocked by adding 250 μL of blocking buffer/well, sealing the plates and incubating at 37°C for 2 hours. Plates were then washed 3 times with PBS/Tween as described previously. Standard was reconstituted in ddH$_2$O to a concentration of 4.86 ng/mL. Standards (see ***Appendix C*** for concentrations used) and samples were then added in duplicate to wells. Standards were diluted in blocking buffer and 100 μL was added to each well. Stimulated samples were added (25 μL) and diluted with 75 μL of blocking buffer whereas 50 μL of unstimulated samples were diluted with 50 μL of blocking buffer so that all wells contained equal volumes. The differences in amount of supernatant added were accounted for in final calculations. Plates were then sealed, incubated for 2 hours at 37°C and washed 4 times with wash buffer.

Biotinylated anti-cytokine detecting antibody was diluted to 1.25 μg/mL in blocking buffer and 100 μL was added to each well. Plates were sealed, incubated at 37°C for 1 hour and washed 4 times with wash buffer. Horseradish peroxidase-streptavidin was diluted 1:10000 in blocking buffer and 100 μL/well was added, plates were sealed, incubated at 37°C for 30 minutes, and washed 4 times with wash buffer. Tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich Inc., St. Louis, MO) was thawed immediately prior to use and was added (100 μL) to each well. Plates were incubated at room temperature for approximately 10 minutes at which time 100 μL of stop solution was added. The optical density was read at 450 nm on a plate reader (Model EL309; Bio-tek Instruments Inc., Burlington, VT).

H) Tumor Necrosis Factor Alpha Production

Supernatants were prepared and frozen at -70°C as described for IL-2. Concentrations of TNF-α were determined via a colorimetric ELISA assay. Antibodies and standards were purchased from R&D Systems, Minneapolis, MN. Purified anti-cytokine capture antibody was diluted to 2 μg/mL in PBS (see ***Appendix C*** for composition of all solutions in this protocol) and added (100 μL) to each well of Immulon® 2 HB high binding flat bottom microtiter plates (Dynex Technologies Inc., Chantilly, VA). Plates were sealed, incubated overnight at room temperature, and washed 3 times with wash buffer. Each wash involved adding 200 µL of wash buffer, gently shaking for 1 minute, inverting and blotting the plate on an absorbent surface. Non-specific binding was blocked by adding 250 µL of blocking buffer/well, sealing the plates and incubating at room temperature for 1 hour. Plates were then washed 3 times with wash buffer as described previously. Standard was reconstituted in diluent to various concentrations (see ***Appendix C*** for concentrations used). Standards and samples were then added (100 µL) in duplicate to wells. Plates were then sealed, incubated for 2 hours at room temperature and washed 3 times with wash buffer.

Biotinylated anti-cytokine detecting antibody was diluted to 200 ng/mL in diluent and 100 µL was added to each well. Plates were sealed, incubated at room temperature for 2 hours and washed 3 times with wash buffer. Avidin-horseradish peroxidase was diluted to 0.5 µL/mL in Blocking Buffer/Tween and 100 µL/well was added, plates were sealed, incubated at room temperature for 30 minutes, and washed 8 times with PBS/Tween. ABTS Substrate Solution was thawed 20 minutes prior to use. 10 µL of 30% H_2O_2/ 11 mL of substrate was added and vortexed. 100 µL of this solution was added to each well. Plates were incubated at room temperature for 30 minutes and the optical density was read at 405 nm on a plate reader (Model EL309; Bio-tek Instruments Inc., Burlington, VT).

I) Nitric Oxide Production

A 4000 µmol/L stock solution of $NaNO_2$ was prepared (27.6 mg $NaNO_2$ + 100 mL ddH_2O) and diluted to 1000 µmol/L with CCM, 4% FCS (v/v) and 2.5 µmol/L 2-mercaptoethanol. From this stock solution, a standard curve was prepared to give concentrations of 100, 80, 60, 50, 40, 30, 20, and 10 µmol/L. A working solution of Griess reagent was prepared by mixing equal volumes of the following two solutions:

Solution X- 0.5 g sulfanilimide was dissolved in 10 mL ddH_2O and 6 mL 85% phosphoric acid q.s to 100 mL with ddH_2O.

Solution Y- 0.05 g N-(1-Naphthyl)ethyl-edediamine in 100 mL ddH_2O.

The CCM as described above was added as a blank to the first well (100 µL) on each non-sterile flat-bottom plate (Fisher, Edmonton, AB). Each standard and sample was then added to wells in triplicate (100 µL). Subsequently, 100 µL of Griess reagent was added to all wells and plates incubated for 10 minutes at room temperature. Plates were then read at 540 nm on an automatic plate reader (Model EL309; Bio-tek Instruments Inc., Burlington, VT). Nitric oxide concentrations for each sample could be calculated from the standard curve.

J) Adipose Triglyceride Analysis

Duplicate samples (100 mg) were taken from the epidydymal fat pads of each male rat. Lipid was extracted by the Folch method (Folch *et al.*, 1957). Briefly, 3 mL of 2:1 chloroform:methanol was added, the sample was homogenized by a polytron (Kinematika GmbH, Brinkmann Instruments, ON) followed by the addition of 750 µL of 0.05% $CaCl_2$. Each sample was then vortexed and left to separate overnight at 4°C. The next day, the top layer was aspirated and the bottom layer transferred to a clean weighed tube. Samples were then dried under $N_2(g)$. Dried samples were weighed to determine lipid mass. One mL of chloroform was then added to redissolve the sample. An appropriate aliquot to provide 5 mg of lipid was then transferred to a clean methylation vial. Sample aliquots were spotted on redi/plate silica gel G plates (Fisher Scientific Inc, Nepean, ON) which had been heat activated at 110°C for 1 hour. Spotted plates were run in a 80:20:1 petroleum ether: diethyl ether: acetic acid (v/v/v) solvent system in a 25 x 25 cm chamber at room temperature. Plates were dried, sprayed with 0.1% (w/v) 8-anilino-1-naphthalene sulfonic acid (ANSA), and TG bands were identified under ultraviolet light. Bands were scraped and added to methylation vials to which 20 µg of C19:0 internal standard had previously been added and dried under $N_2(g)$.

Samples with internal standards were saponified by adding 2 mL 0.5M KOH in methanol and heating at 110°C for 1 hour on a sandbath. After cooling for 5 minutes, 2 mL each of hexane and 14% boron trifluoride (w/w) in methanol were added. Methylation was carried out for 30 minutes at room temperature with the samples being continuously shaken. One mL of ddH_20 was added to stop the reaction, the sample was vortexed and left overnight at 4°C to allow separation of phases.

The following day, the upper hexane phase was removed and transferred to gas chromatography (GC) vials. The resulting fatty acid methyl esters (FAME) were then dried down. Analysis and identification of the FAME was carried out by gas liquid chromatography (GLC). Analyses were carried out on a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d. x 0.2 μm film thickness; Supelco Inc, Bellefonte, PA) by dissolving samples in hexane and setting the column conditions as follows:

injector port: 250°C   detector temperature: 270°C
gas carrier: helium   column pressure: 50 psi
100: split mode

Samples were eluted over 110 minutes using temperatures from 130°C to 225°C. By this method, all saturated, monounsaturated and polyunsaturated FAME ranging from C14 to C24 were separated. As demonstrated in ***Figure 3.2***, several isomers of CLA were also separated by this column.

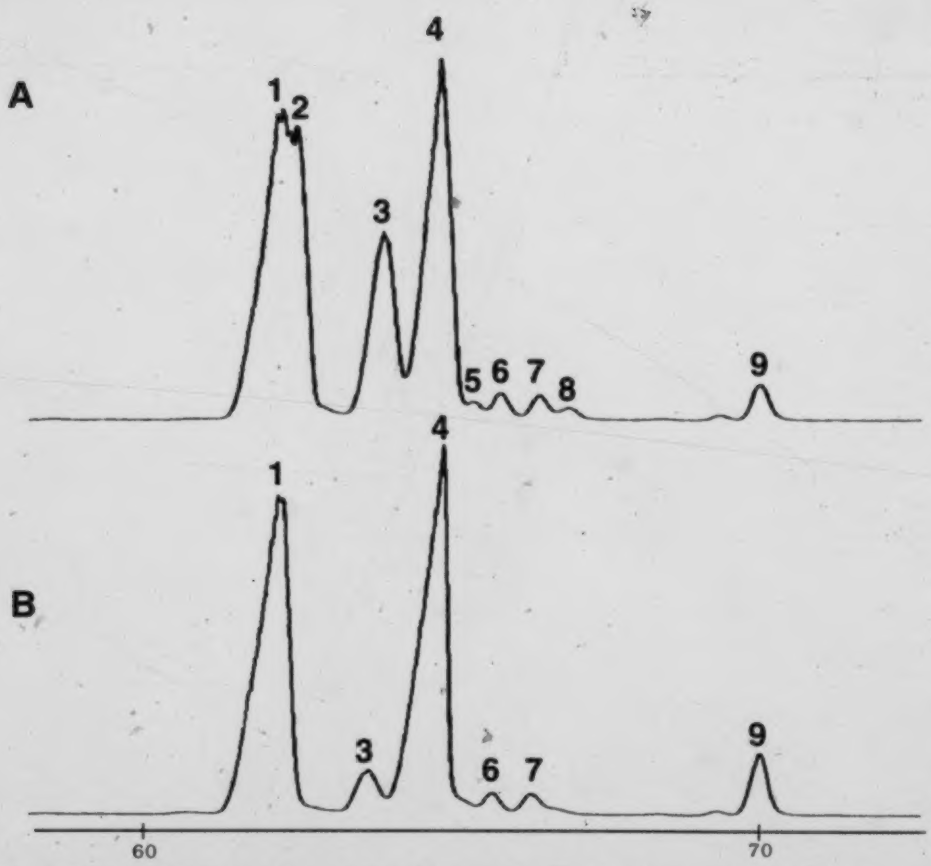


Figure 3.2. ***GLC Expansion of CLA Isomer Peaks.*** *Gas chromatographic expansions of CLA isomers from a SP-2560 fused silica GLC column. Commercially available CLA contains 9 peaks (panel A). As a percentage of the total mixture, peaks 1,2,3,4, 5-8 and 9 correspond to 31.5±4.5, 11.3±1.6, 17.3±0.8, 32.2±0.7, 3.8±1.1 and 3.8±1.7%, respectively (n=2). CLA produced from LA derived from safflower oil was separated into 6 peaks (panel B). As a percentage of the CLA isomers, peaks 1,3,4,6,7 and 9 correspond to 45.0±0.7, 3.1±0.7, 46.1±1.0, 0.9±0.04, 1.4±0.1, and 3.5±0.7%, respectively (n=4). Peaks 1,4 and 9 corresponding to 9c,11t-, 10t,12c-, and 9t,11t-18:2, respectively, were identified in the safflower derived mixture.*
(personal communication, D.W.L. Ma, 1998)

K) Splenocyte Phospholipid Analysis

A modified Folch method (Folch *et al.*, 1957) was performed on individual rat stimulated and unstimulated cultured splenocytes to extract lipid from these cells. Briefly, 1.6 mL of 0.1M KCl was added to tubes containing frozen splenocyte pellets.

This was vortexed and the suspended cells were transferred to a clean 15 mL methylation tube. 0.8 mL methanol, 2.0 mL 1:1 chloroform: methanol, 2.7 mL chloroform, and 2.5 mL 2:1 chloroform: methanol were added sequentially with vortexing after each. Samples were then capped and vortexed for 1 minute and left overnight at 4°C to separate. The next day, the bottom layer was removed, transferred to a clean methylation vial, and dried under $N_2(g)$. An additional 1 mL of chloroform was added to the original tube, vortexed and the bottom layer again transferred once the phases had separated. Chloroform (100 µL) was added to the dried sample which was then stored at -70°C until they were run on silica gel H plates (Fisher Scientific Inc, Nepean, ON).

H plates were heat activated at 110°C for 1 hour prior to spotting phospholipid samples. Samples were run in a 30: 9: 25: 6: 18 (by volume) chloroform: methanol: 2-propanol: 0.25% KCl: triethylamine solvent system in a 13 x 13 cm chamber at room temperature for approximately 45 minutes. Plates were dried, sprayed with 0.1% (w/v) ANSA, and bands were identified under ultraviolet light. Bands were then scraped and added to methylation vials. Phosphatidylinositol (PI), sphingomyelin (SM) and phosphatidylserine (PS) bands were stored at -70°C for later methylation and analysis. Phosphatidylcholine and phosphatidylethanolamine (PE) were methylated immediately using 0.75 mL 14% boron trifluoride (w/w) in methanol and 1.5 mL of distilled hexane. Methylation proceeded for 30 minutes at room temperature with the samples being continuously shaken. One mL of ddH_20 was added to stop the reaction, the sample was vortexed and left overnight at 4°C to allow separation of phases. The upper layer was removed, transferred to a GC vial and dried under $N_2(g)$. An additional 0.5 mL of distilled hexane was added, vortexed and allowed to separate. The upper layer was again removed and added to the GC vial. Dried samples were flushed with nitrogen and stored at -70°C until analysis. Analyses were carried out on a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d. x 0.2 µm film thickness; Supelco Inc, Bellefonte, PA) by dissolving samples in hexane and setting the column conditions as follows:

injector port: 250°C detector temperature: 270°C
gas carrier: helium column pressure: 50 psi
40: split mode

Samples were eluted over 110 minutes using temperatures from 130°C to 225°C. By this method, all saturated, monounsaturated and polyunsaturated FAME ranging from C14 to C24 were separated. Several isomers of CLA were also separated by this column (see ***Figure 3.2***).

L) Body Composition

Rat carcasses were placed in individual pressure cookers with 1 L of water and allowed to cook for 2.5 hours at 15-20 psi. The carcass and remaining water were transferred into a blender and blended until a homogenous mixture was obtained. The mixture was poured into a 500 mL foil pan and allowed to freeze at -30°C overnight. Each frozen samples was freeze dried and the resulting sample was blended again to obtain a homogenous dry mixture. This mixture was stored at -20°C for later analysis. All analyses were performed in duplicate or triplicate and all weighing was performed to the nearest 0.0001g.

Lipid Analysis- The percentage of total body composition as lipid was determined according to standard chemical analysis procedures of the Association of Official Analytical Chemists (AOAC, 1980). Empty extraction beakers were initially weighed. Approximately 2 g of freeze dried mixture for each animal was placed in filter paper in an aluminum thimble and covered with glass wool. Thimbles were placed in the extraction apparatus and lipid was extracted for four hours with approximately 40 mL of petroleum ether. Beakers were then cooled, the thimbles were replaced with ether collection beakers and burners were turned on again in order to collect the ether. When the residue in the extraction beaker appeared to be thicker (i.e. ether had been removed), beakers were again cooled, covered in perforated aluminum foil and placed in an oven at 110°C for 45 minutes to evaporate any remaining ether. Beakers were then allowed to cool and weights were taken. All animals were done in duplicate and values obtained were considered acceptable when the percent range was less than or equal to 5% of the mean value as determined by the equation:

% range= ((higher value- lower value) x 100)/ mean

If the percent range was ≥5%, a third replicate was run and the two closest were used to obtain an average. This resulted in all lipid values having a percent range less than or equal to 5% of the mean value.

Protein Analysis- All protein analyses were done on the LECO FP-428 nitrogen analyzer (Leco Corporation, St. Joseph, MI). Protein values were determined using the standard factor of 6.25 x nitrogen value. Duplicates or triplicates (of which the closest two were chosen) from each animal were taken and values obtained were considered acceptable when the percent range was less than or equal to 3% of the mean value as described previously. All animals had a percent range within acceptable limits.

Dry Matter and Ash Analysis- Dry beakers were weighed and approximately 2 g of freeze dried sample for each animal was added in duplicate. One empty beaker was used as a blank for correction factor purposes. Beakers were covered with perforated aluminum foil and placed in an oven at 110°C overnight. Beakers were then cooled in a dessicator and weighed. Dry matter (DM) was determined by the equations:

correction factor 1= initial blank weight - final blank weight

% DM= (final beaker weight - empty beaker weight - correction factor 1) x 100/ initial sample weight

Ash was determined by placing these same beakers in an oven overnight at 500°C, followed by cooling in a dessicator and weighing. Percent ash was determined using the equations:

correction factor 2= blank weight after drying at 110°C - blank weight following ashing at 500°C

% Ash= (final ashed beaker weight - empty beaker weight - correction factor 2) x 100/ initial sample weight

If the percent range of the sample duplicates was ≥5%, a third replicate was run and the closest two replicates were used to obtain a mean value. All body composition values for water, ash, DM, protein and lipid were then corrected for moisture loss during carcass homogenization and freeze drying by monitoring changes in sample weight due to changes in water content throughout the previously described procedures. The equations used are outlined in ***Appendix D***. Body composition values were calculated on a full body weight basis rather than a dry weight basis. The rationale for this is also outlined in ***Appendix D***.

M) Statistical Analyses

Body weight and food intake were analyzed by three-way ANOVA using P/S ratio, CLA supplementation and sex as factors. Natural Killer Cytotoxicity and Proliferation were analyzed by split plot ANOVA to account for high variation between animals. Phenotypic analysis, cytokine and nitric oxide production, adipose TG and immune PL data were analyzed by two-way ANOVA using P/S ratio and CLA supplementation as the two treatments. Body composition was similarly analyzed using male rats only to remove sex as a factor and to provide an adequate "n" value in each group. Unless otherwise stated, the statistical significance level was set at $p<0.05$ and differences between groups were identified by LSMEANS. All statistical analyses were conducted using SAS (Version 6, SAS Institute, Cary, NC).

III. RESULTS

A) Body Weight

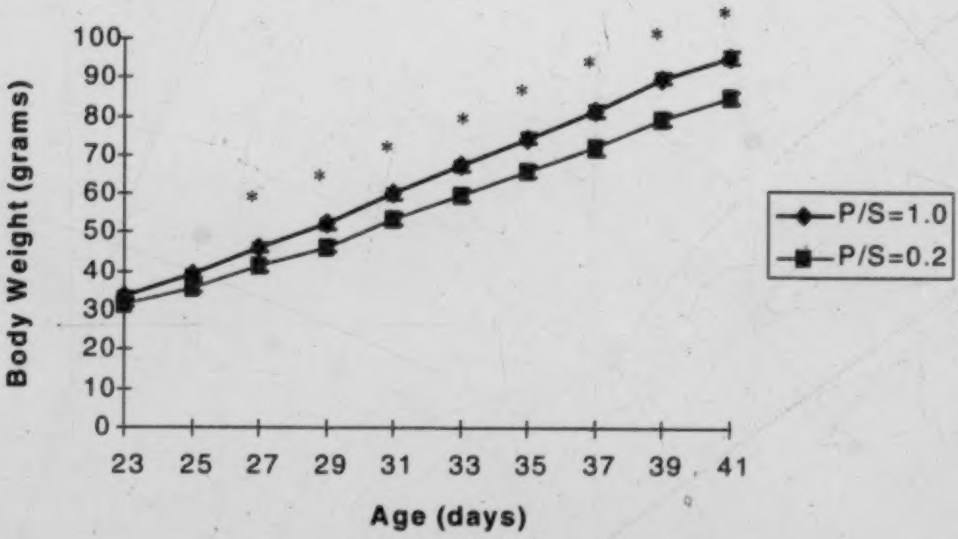


Figure 3.3. Body Weight Measurement During the Feeding Period. *CLA groups (supplemented versus non-supplemented) were combined as there were no differences. Values represent mean ± SEM, n=20 in each treatment group. "*" indicates a significant difference between high and low P/S diets ($p<0.05$).*

The dietary P/S ratio had a significant effect ($p<0.05$) on body weight. Rats fed the high P/S diet demonstrated an increased body weight compared to those fed the low P/S diet. This difference was significant after only six days of diet treatment ($p<0.02$). The addition of CLA to the diets did not significantly affect body weight.

B) Food Intake

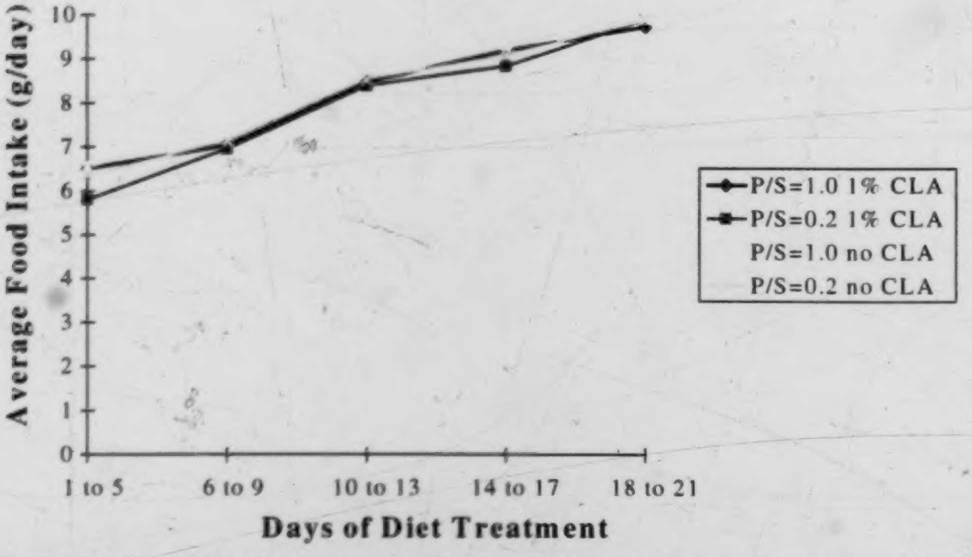


Figure 3.4. Dietary Intake During the Feeding Period. *Values represent the mean ± SEM with n=10 for each treatment group..*

Food intake did not differ significantly between any of the diet treatments at any time during the three week feeding period.

C) Spleen Weight

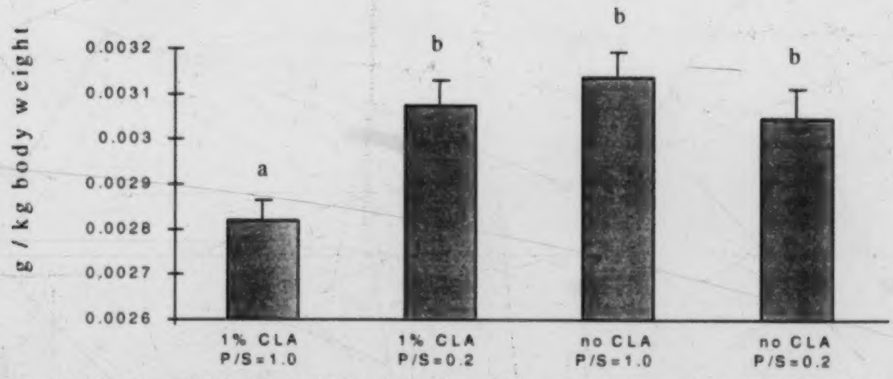
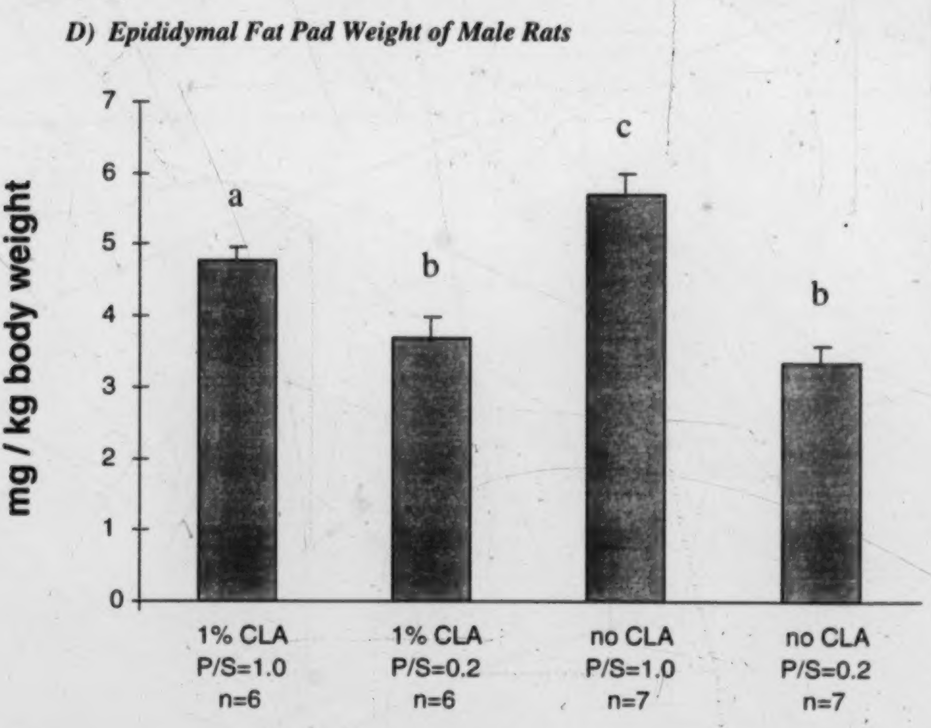


Figure 3.5. Spleen Weight Per Body Weight. *Bars represent mean ± SEM (n=10/ treatment group). Bars that do not share the same letter are significantly different ($p<0.05$).*

Feeding CLA had a significant effect on spleen weight/body weight. Rats fed CLA in a high P/S diet had a significantly lower spleen weight per body weight ($p<0.01$) than any of the other three groups.



D) Epididymal Fat Pad Weight of Male Rats

Figure 3.6. Epididymal Fat Pad Weight Per Body Weight. *Bars represent mean ± SEM. Bars that do not share the same letter are significantly different ($p<0.05$).*

The diet P/S ratio significantly affected epididymal fat pad weight/body weight (p=0.0001). Rats fed a high P/S diet had higher fat pad weight/body weight than those fed the low P/S diets, similar to trends seen for body weight. There was also a diet P/S and CLA interaction (p=0.03) Animals fed a high P/S diet with CLA supplementation exhibited lower epididymal fat pad weights/body weight than rats fed a high P/S diet and not receiving CLA (p=0.03). There was no effect of CLA supplementation on epididymal fat pad weight when provided in a low P/S diet.

E) Natural Killer Cytotoxicity

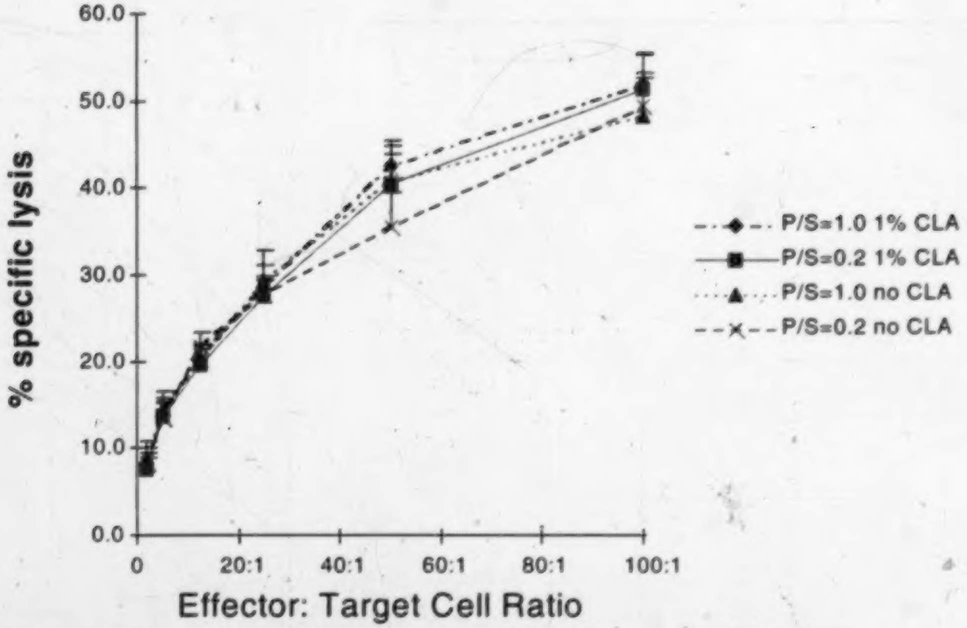


Figure 3.7. Natural Killer Cytotoxicity at Various Effector:Target Ratios. *Points represent mean ± SEM (n=10 / treatment group).*

There were no differences in natural killer cytotoxic activity against YAC-1 cells at all the effector: target cell ratios studied. Similarly, no effects of P/S ratio (p=0.13) or CLA supplementation (p=0.30) were found when NK cytotoxocity was expressed as lytic units (the number of NK cells ($x10^3$) required to lyse 20% of target cells). An average value of 1.56 ± 0.28 lytic units was obtained for all animals studied.

F) Splenocyte Phenotyping

i) Freshly Isolated Splenocytes

	ox19+	**w3/25+**	**ox8+**	**ox12+**	**ox42+**	**3.2.3+**
P/S=1.0 + CLA n ≥ 9	43 ± 2	22 ± 3	20 ± 3	35 ± 3	10 ± 1	10 ± ^{2ab}
P/S=0.2 + CLA n ≥ 9	48 ± 2	27 ± 3	20 ± 1	32 ± 3	11 ± 1	11 ± 2^{b}
P/S=1.0 - CLA n ≥ 8	47 ± 2	25 ± 3	21 ± 2	33 ± 2	9.0 ± 0.8	6.6 ± 0.5^{a}
P/S=0.2 - CLA n ≥ 8	46 ± 3	24 ± 2	21 ± 2	31 ± 2	11 ± 2	10 ± 1^{ab}

Table 3.2. Phenotypes of Freshly Isolated Splenocytes. *Values represent the mean percentage of spleen lymphocyte population expressing each marker ± SEM. Values in a column for each marker that do not share the same letter are significantly different ($p<0.05$).*

There were no significant differences due to P/S ratio or CLA supplementation in the percentage of spleen lymphocytes as ox19+, w3/25+, ox8+, ox12+, ox42+, or 3.2.3 cells. From the LSMEANS, the high P/S diet group which was not CLA-supplemented demonstrated a decrease in 3.2.3+ cells compared to the low P/S CLA-supplemented group (p=0.02) however this did not result in a significant change in lytic units or natural killer cytotoxicity (see ***Figure 3.6***)

	ox19+ w3/25+	ox19+ ox8+
P/S=1.0 + CLA n ≥ 9	20 ± 3	18 ± 2
P/S=0.2 + CLA n ≥ 9	25 ± 3	18 ± 2
P/S=1.0 - CLA n ≥ 8	24 ± 3	21 ± 3
P/S=0.2 - CLA n ≥ 8	22 ± 2	18 ± 2

Table 3.3. CD4 and CD8 cells that are CD5 positive in Freshly Isolated Splenocytes. *Values represent the mean percentage of spleen lymphocyte population expressing each marker ± SEM.*

There were no significant differences among dietary treatment groups in the proportion of CD4+ and CD8+ T cells.

ii) Splenocytes Stimulated with Concanavalin A and Cultured for 48 hours

	ox26+	ox39+	ox19+	w3/25+	ox8+	ox12+	ox42+	w3/25+/ox8+
P/S=1.0 + CLA n=10	24 ± 4^{ab}	20 ± 4^{ab}	34 ± 4	29 ± 4	26 ± 5	50 ± 6	24 ± 5	1.25 ± 0.10
P/S=0.2 + CLA n=10	33 ± 5^{b}	23 ± 5^{b}	34 ± 4	29 ± 5	28 ± 6	53 ± 5	24 ± 6	1.10 ± 0.11
P/S=1.0 - CLA n=10	23 ± 5^{ab}	16 ± 4^{ab}	32 ± 4	21 ± 3	16 ± 3	41 ± 5	14 ± 3	1.50 ± 0.18
P/S=0.2 - CLA n=9	18 ± 4^{a}	11 ± 4^{a}	25 ± 4	18 ± 3	20 ± 6	41 ± 6	16 ± 3	1.31 ± 0.27
p-value	0.05	0.06	NS	NS	NS	NS	NS	NS

Table 3.4. Phenotypes of Splenocytes Cultured for 48 Hours and Stimulated with Concanavalin A. *Values represent the mean percentage of spleen lymphocytes expressing each marker ± SEM. Values for each marker that do not share the same letter are significantly different at the p-value indicated as determined by LSMEANS.*

In examining the LSMEANS, rats fed low P/S diets and dietary CLA displayed an increase in the percentage of splenocytes expressing ox26 and ox39 compared to their non-supplemented counterparts. There were no differences among diet treatment groups in the percentage of spleen lymphocytes expressing ox19, w/25, ox8, ox12 or ox42. There were also no differences in the CD4:CD8 (w/25:ox8) ratio.

	ox26+ox19+	ox26+w3/25+	ox26+ox8+	ox26+ox12+	ox26+ox42+
P/S=1.0 + CLA n= 8	13 ± 3^{a}	12 ± 2^{a}	11 ± 3^{ab}	14 ± 4^{a}	10 ± 2^{ab}
P/S=0.2 + CLA n≥ 9	23 ± 4^{b}	22 ± 5^{b}	19 ± 5^{b}	29 ± 5^{b}	14 ± 4^{b}
P/S=1.0 - CLA n= 9	16 ± 3^{ab}	11 ± 2^{a}	8.1 ± 1.9^{a}	16 ± 4^{a}	7.0 ± 1.3^{ab}
P/S=0.2 - CLA n= 8	9.3 ± 2.7^{a}	8.5 ± 2.1^{a}	4.1 ± 1.2^{a}	10 ± 4^{a}	5.8 ± 1.6^{a}
CLA effect	p=0.15	p=0.02	p=0.01	p=0.05	p=0.04
PS*CLA effect	p=0.02	p=0.06	p=0.09	p=0.02	p=0.34

Table 3.5. Phenotypes of Splenocytes Stimulated with Concanavalin A, Cultured for 48 Hours and Expressing the Transferrin Receptor (ox26+). *Values represent the mean percentage of spleen lymphocytes expressing each marker ± SEM. Values for each marker that do not share the same letter are significantly different ($p<0.05$) as determined by LSMEANS.*

Rats fed a low P/S diet supplemented with CLA consistently demonstrated a higher percentage of ox19+, w3/25+, ox8+, ox12+, and ox42+ cells expressing the activation marker (ox26+) than rats fed the low P/S diet without CLA supplementation.

	ox39+ox19+	ox39+w3/25+	ox39+ox8+	ox39+ox12+	ox39+ox42+
P/S=1.0 + CLA n= 8	11 ± 3^{ab}	12 ± 3^{ab}	9.2 ± 3.1^{ab}	11 ± 4^{ab}	9.3 ± 3.4^{ab}
P/S=0.2 + CLA n≥ 9	17 ± 4^{b}	16 ± 4^{b}	15 ± 5^{b}	19 ± 6^{b}	17 ± 6^{b}
P/S=1.0 - CLA n≥ 8	11 ±3^{ab}	8.6 ± 2.3^{ab}	3.8 ± 0.9^{a}	7.0 ± 2.0^{a}	3.7 ± 1.0^{a}
P/S=0.2 - CLA n= 8	4.5 ± 1.3^{a}	4.6 ± 1.7^{a}	3.6 ± 1.5^{a}	5.1 ± 2.0^{a}	3.1 ± 1.0^{a}
CLA effect	p=0.05	p=0.02	p=0.02	p=0.04	p=0.02
PS*CLA effect	p=0.04	p=0.24	p=0.39	p=0.23	p=0.30

Table 3.6. Phenotypes of Splenocytes Stimulated with Concanavalin A, Cultured for 48 Hours, and Expressing the Interleukin-2 Receptor (ox39+). *Values represent the mean percentage of spleen lymphocytes expressing each marker ± SEM. Values for each marker that do not share the same letter are significantly different ($p<0.05$).*

Rats fed a low P/S diet supplemented with CLA consistently demonstrated a higher percentage of ox19+, w3/25+, ox8+, ox12+, and ox42+ cells expressing the interleukin-2 receptor (ox39+) than non-CLA supplemented rats fed the low P/S diet.

G) Proliferation

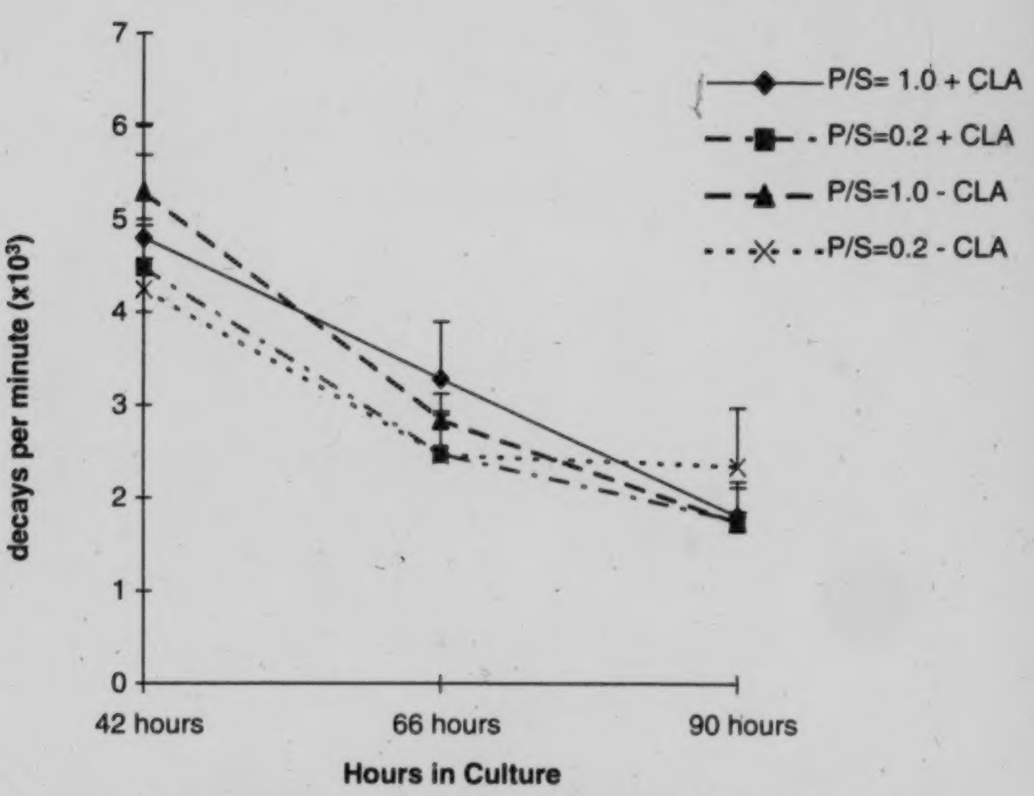


***Figure 3.8.** ^{3}**H-Thymidine Incorporation into Unstimulated Cultured Splenocytes.** Values represent mean ± SEM, n≥9 in each treatment group.*

i) Unstimulated splenocytes - There were not significant differences between diets at any time point as determined by one-way ANOVA.

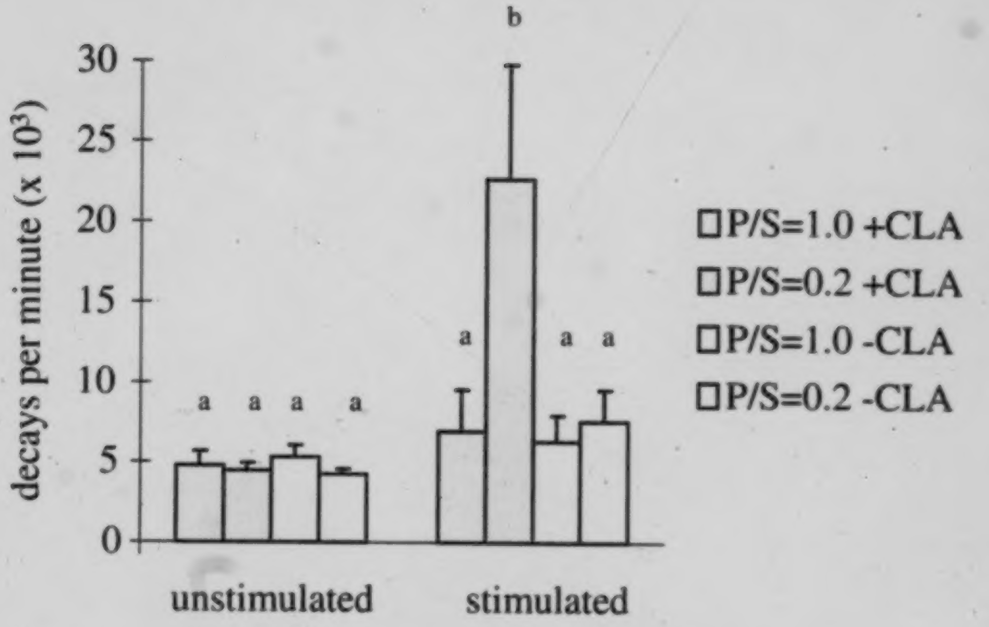


Figure 3.9. Splenocytes Cultured for 42 Hours with or without Concanavalin A Stimulation. *Values represent mean ± SEM, $n \geq 9$ in each treatment group. Different letters indicate significant differences between diet treatments for stimulated and unstimulated cells ($p<0.05$).*

There were no significant differences between stimulated and unstimulated proliferation for any diet treatment group with the exception of cells from the low P/S, CLA-supplemented group. Cells from this group demonstrated an increase in proliferation with Concanavalin A stimulation at 42 hours compared to unstimulated cells ($p<0.0001$) and all the other diet groups.

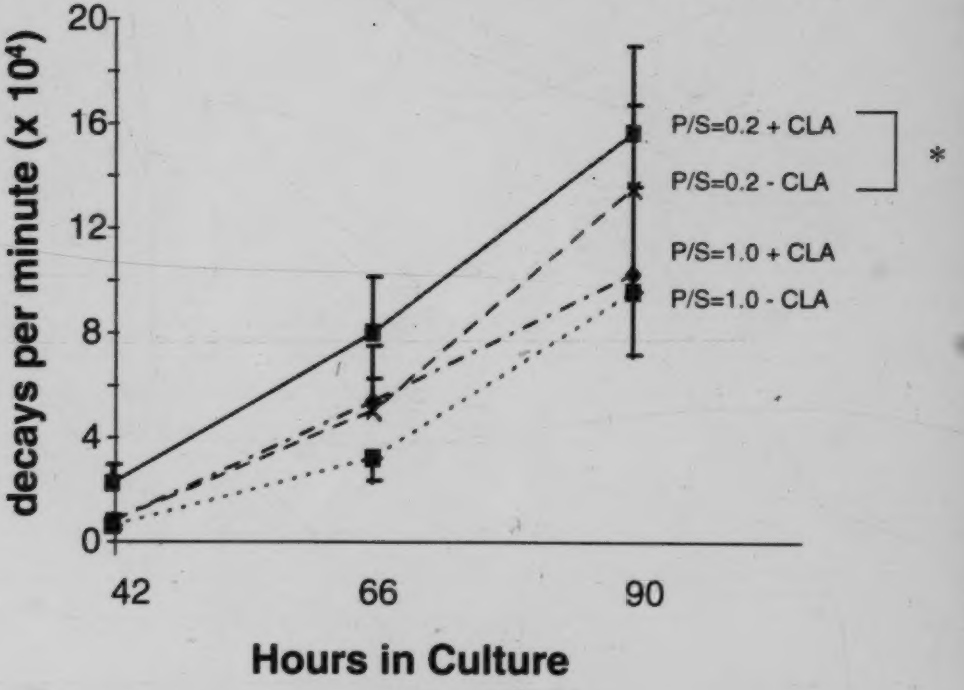


Figure 3.10. ***^{3}H-Thymidine Incorporation into Cultured Splenocytes Stimulated with Concanavalin A.*** *Values represent mean ± SEM, n≥9 in each treatment group. "*" indicates significant differences between diet treatments ($p<0.05$).*

ii) Concanavalin A-stimulated Splenocytes**-** Statistical analysis was carried out by split plot ANOVA on the latter two time points. These two points were used as no changes were generally noted in proliferation compared to unstimulated cells after 42 hours in culture ($p>0.05$, see ***Figure 3.9***) with the exception of the low P/S supplemented with CLA. Both P/S ratio ($p=0.01$) and CLA ($p=0.05$) significantly enhanced proliferation. Rats fed a low P/S diet demonstrated increased proliferation compared to those fed a high P/S diet (statistics not indicated on figure). As well, for the low P/S diets, CLA supplementation enhanced splenocyte proliferation compared to rats not supplemented with CLA ($p<0.04$).

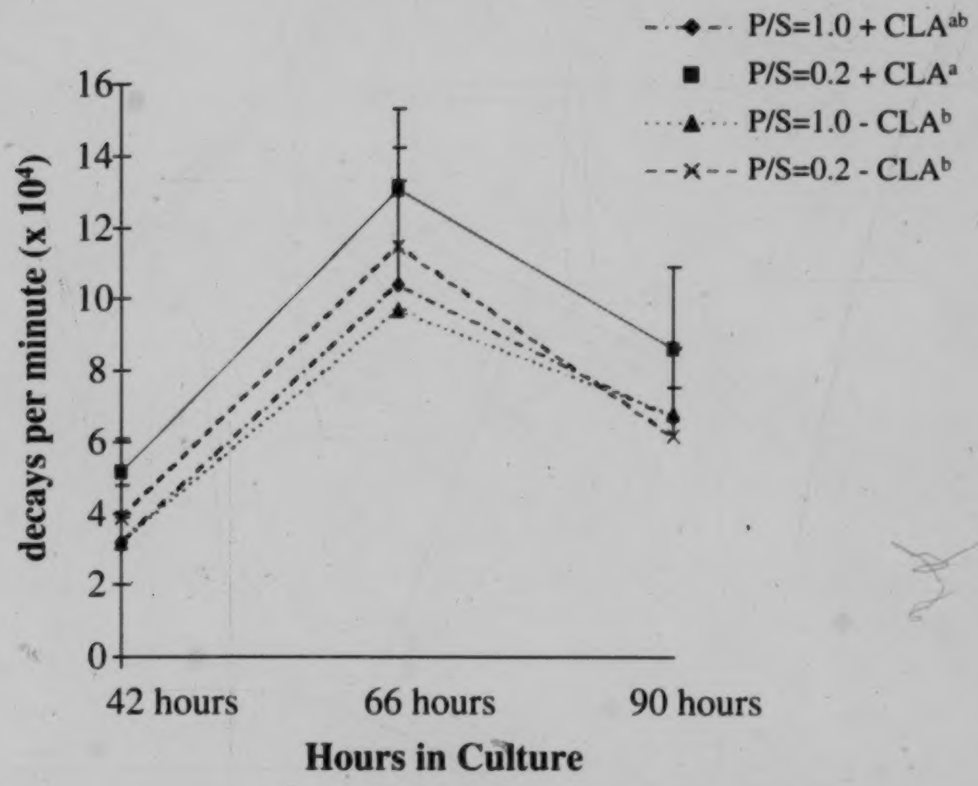


Table 3.11. ^{3}H-Thymidine Incorporation into Cultured Splenocytes Stimulated with Phorbol Myristate Acetate + Ionomycin. *Values represent mean ± SEM, n≥9 in each treatment group. Different letters on the diet groups in the legend indicate significant differences between diet treatments ($p<0.05$).*

iii) PMA + Iono-stimulated Splenocytes- Whereas P/S ratio did not affect proliferation ($p=0.20$), rats fed CLA demonstrated enhanced proliferation compared to those that were not supplemented ($p=0.05$). However, the higher response to CLA appeared to only occur in the animals fed the low P/S diet, similar to the diet effect seen in the Con A response.

H) Interleukin-2 Production

There was no IL-2 production by unstimulated splenocytes cultured for 48 hours by any of the diet treatment groups.

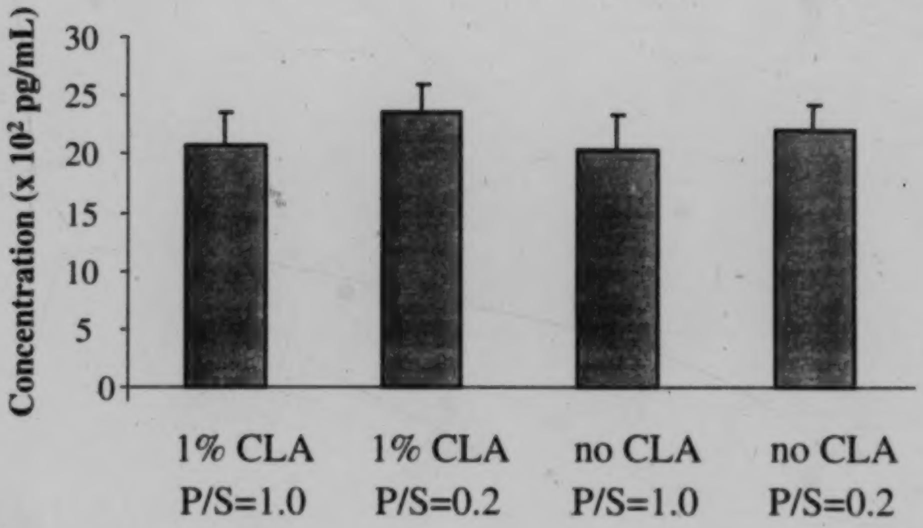


Figure 3.12. Interleukin-2 Production by Splenocytes Stimulated with Concanavalin A for 48 hours. *Bars represent mean ± SEM (n=10 / treatment group).*

Despite differences in the proliferative response, IL-2 production did not differ significantly among any of the diet treatment groups following 48 hour culture with Con A stimulation.

I) Nitric Oxide Production

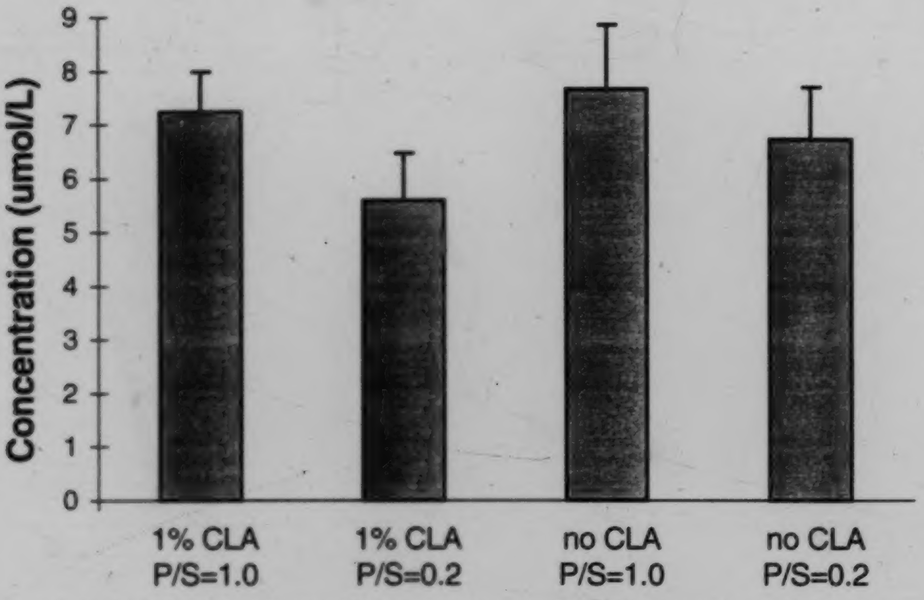


Figure 3.13. Nitric Oxide Production by Unstimulated Splenocytes. *Bars represent mean ± SEM (n=10 / treatment group).*

Nitric oxide production by unstimulated splenocytes did not differ significantly among any of the diet treatment groups following 48 hour of culture.

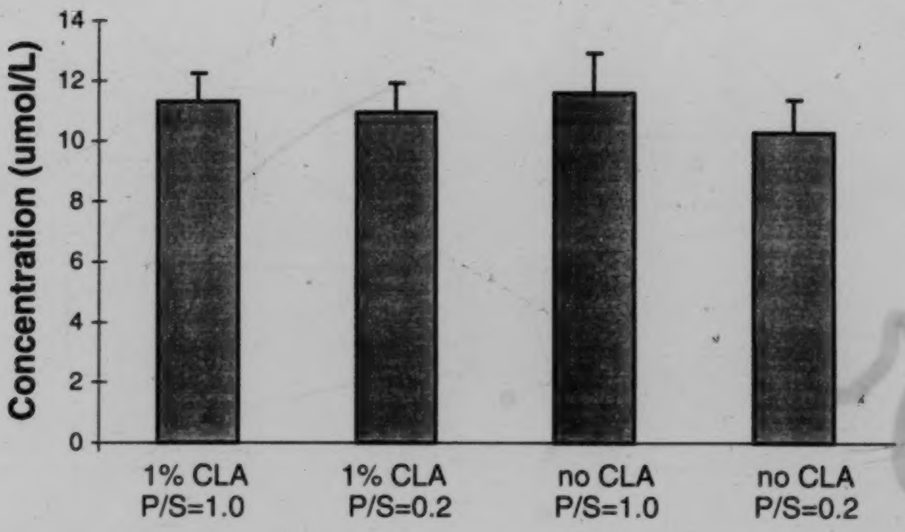


Figure 3.14. Nitric Oxide Production by Splenocytes Stimulated with Concanavalin A for 48 hours. *Bars represent mean ± SEM (n=10 / treatment group).*

Nitric oxide production by splenocytes did not differ significantly among any of the diet treatment groups following 48 hour culture with Con A stimulation.

J) Interferon Gamma Production

There was no IFN-γ production by unstimulated splenocytes cultured for 48 hours by any of the diet treatment groups.

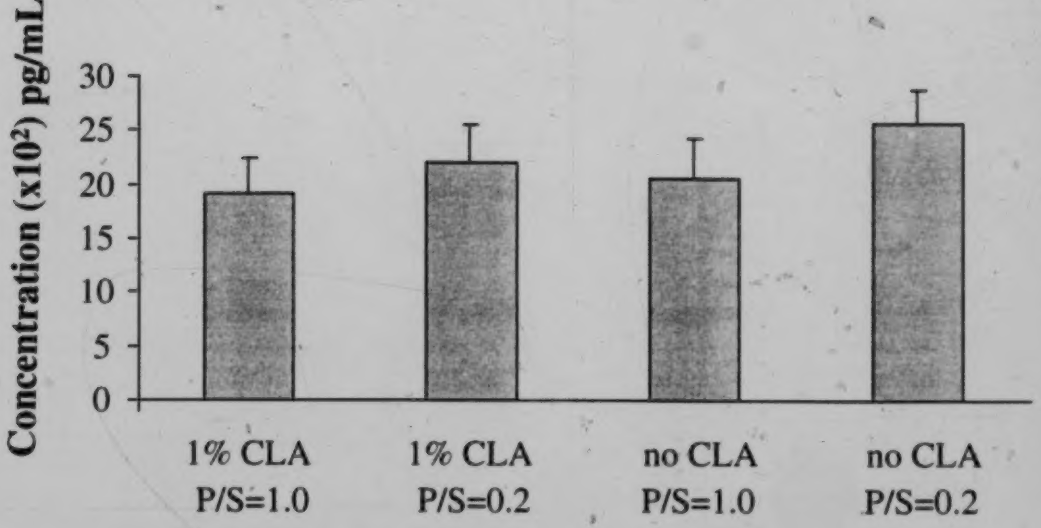


Figure 3.15. Interferon Gamma Production by Splenocytes Stimulated with Concanavalin A for 48 hours. *Bars represent mean ± SEM (n=10 / treatment group).*

Interferon gamma (IFN-γ) production by splenocytes did not differ significantly among any of the diet treatment groups following 48 hour culture with Con A stimulation.

K) Tumor Necrosis Factor Alpha Production

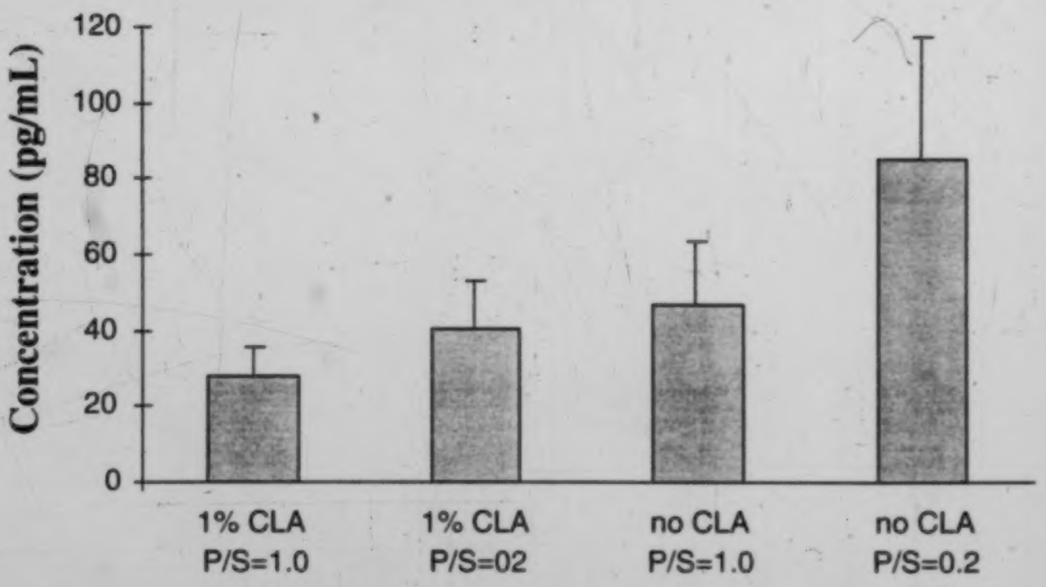


Figure 3.16. Tumor Necrosis Factor Alpha Production by Unstimulated Splenocytes. *Bars represent mean ± SEM (n≥8 / treatment group).*

Tumor necrosis factor alpha (TNF-α) production by splenocytes did not differ significantly among any of the diet treatment groups.

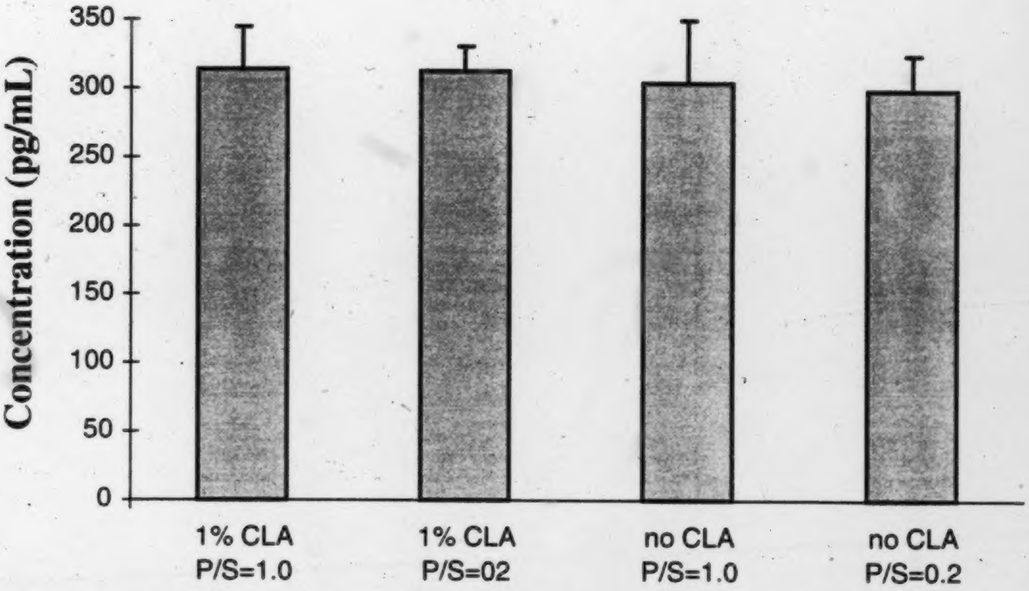


Figure 3.17. Tumor Necrosis Factor Alpha Production by Splenocytes Stimulated with Concanavalin A for 48 Hours. *Bars represent mean ± SEM (n≥9 / treatment group).*

TNF-α production by splenocytes did not differ significantly among any of the diet treatment groups following 48 hour culture with Con A stimulation.

L) Fatty Acid Composition of Epididymal Fat Pad Triglycerides

Fatty Acid	Diet			
	P/S=1.0 + CLA	*P/S=0.2 + CLA*	*P/S=1.0 - CLA*	*P/S=0.2 - CLA*
Saturates				
12:0	0.32 ± 0.03	0.36 ± 0.03	0.17 ± 0.03	0.30 ± 0.03
14:0	3.40 ± 0.04	4.46 ± 0.03	2.63 ± 0.06	3.80 ± 0.06
16:0	31.6 ± 0.39	35.6 ± 0.6	27.4 ± 0.7	30.0 ± 0.4
17:0	0.37 ± 0.01	0.62 ± 0.01	0.27 ± 0.01	0.45 ± 0.02
18:0	7.82 ± 0.34	10.9 ± 0.3	5.78 ± 0.17	8.09 ± 0.30
19:0	0.61 ± 0.08	0.58 ± 0.06	0.55 ± 0.03	0.55 ± 0.03
Total Saturates	**44.3 ± 0.6**	**52.9 ± 0.6**	**36.6 ± 0.8**	**42.9 ± 0.9**
Monounsaturates				
16:1 (7)	4.18 ± 0.33	4.47 ± 0.23	6.92 ± 0.44	7.10 ± 0.29
18:1 (9)	17.5 ± 0.4	23.6 ± 0.5	22.0 ± 0.4	27.3 ± 0.7
Total Monounsaturates	**21.7 ± 0.7**	**28.0 ± 0.7**	**28.0 ± 1.0**	**34.4 ± 0.9**
Polyunsaturates				
18:2 (6)	29.8 ± 0.8^{a}	11.8 ± 0.4^{x}	33.6 ± 0.8^{b}	19.8 ± 0.6^{y}
18:3 (3)	0.57 ± 0.03^{a}	0.81 ± 0.05^{x}	0.65 ± 0.02^{a}	0.83 ± 0.04^{x}
20:4 (6)	0.05 ± 0.01^{a}	0.15 ± 0.01^{x}	0.01 ± 0.00^{b}	0.02 ± 0.01^{y}
Total CLA	2.86 ± 0.26^{a}	5.65 ± 0.25^{x}	0.27 ± 0.01^{b}	0.50 ± 0.09^{y}
Total Polyunsaturates	**33.3 ± 1.0^{a}**	**18.4 ± 0.7^{x}**	**34.5 ± 0.8^{a}**	**21.2 ± 0.5^{y}**

Table 3.7. Fatty Acid Profile of Male Epididymal Fat Pad Triglycerides as Determined by Gas Liquid Chromatography. *Values represent mean ± SEM with* $n \geq 5$ *for each dietary treatment group. For polyunsaturated fatty acids, different superscripts indicate significant differences between CLA supplemented and non-supplemented animals within the same P/S ratio* ($p<0.05$)*.*

CLA incorporation into adipose tissue of rats fed CLA was significantly higher than those not fed CLA ($p=0.0001$). The level of incorporation of CLA for supplemented rats was significantly affected by P/S ratio ($p=0.0001$). Animals fed the low P/S diet incorporated 5.65±0.25 % (w/w) of total fatty acids as CLA. However, CLA-supplemented rats fed a high P/S diet incorporated only 2.86±0.26% (w/w) of total fatty acids as CLA.

P/S Ratio	**CLA Isomer**				
	c9t11	***t10c12***	***t9t11***	**Other**	***c9t11: t10c12***
1.0	57.0 ± 0.5	36.0 ± 0.2	5.04 ± 0.46	1.98 ± 0.09	1.58 ± 0.02
0.2	58.3 ± 0.7	34.8 ± 0.9	4.76 ± 0.28	2.17 ± 0.07	1.68 ± 0.06

Table 3.8. Each Isomer as a Percent of Total CLA in Epididymal Fat Pads From Rats fed CLA-supplemented Diets. *Values represent mean ± SEM with n≥5 for each dietary treatment group.*

Although the absolute incorporation of CLA differed in animals fed the different diets, the relative incorporation of the two major isomers of CLA was not significantly affected by dietary P/S ratio.

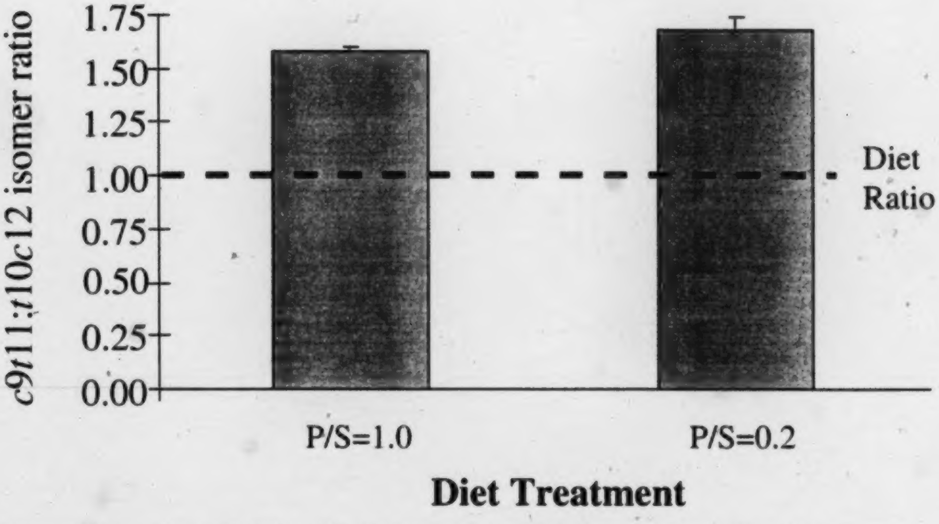


Figure 3.18. Ratio of c9t11:t10c12 Isomers in CLA-Supplemented Rat Adipose Triglycerides versus Diet. *Bars represent mean ± SEM.*

Analysis of dietary CLA indicated that the two major isomers, *c9t*11 and *t*10*c*12, were present in approximately equal proportions (refer to ***Figure B.2*** in ***Appendix B***). In examining the epididymal fat pad triglycerides, it was found that the ratio between these two isomers was higher than the dietary ratio (***Figure 3.18***). Relative to dietary levels, the proportion of *c9t*11 increased and the proportion of *t*10*c*12 decreased (***Table***

3.8 and ***Figure B.2*** in ***Appendix B***).

M) Splenocyte Phospholipid Fatty Acid Analysis

Fatty Acid	Diet			
	P/S=1.0 + CLA	*P/S=0.2 + CLA*	*P/S=1.0 - CLA*	*P/S=0.2 – CLA*
Saturates				
16:0	30.2 ± 1.1^{b}	28.1 ± 2.0^{ab}	32.4 ± 1.8^{b}	25.7 ± 1.4^{a}
17:0	0.47 ± 0.11	0.51 ± 0.15	0.35 ± 0.13	0.53 ± 0.12
18:0	19.9 ± 0.5^{ab}	19.8 ± 0.7^{ab}	21.3 ± 0.8^{b}	19.0 ± 0.8^{a}
Total Saturates	$\mathbf{51.4 \pm 1.0^{b}}$	$\mathbf{48.4 \pm 1.8^{ab}}$	$\mathbf{52.3 \pm 2.1^{b}}$	$\mathbf{45.1 \pm 1.3^{a}}$
Monounsaturates				
16:1	0.62 ± 0.10^{a}	0.75 ± 0.06^{ab}	0.86 ± 0.12^{ab}	1.03 ± 0.18^{b}
18:1	10.9 ± 0.6^{a}	13.5 ± 1.0^{b}	9.51 ± 0.64^{a}	14.2 ± 1.2^{b}
20:1	1.55 ± 0.10^{b}	0.81 ± 0.10^{a}	1.55 ± 0.31^{b}	1.00 ± 0.28^{ab}
Total Monounsaturates	$\mathbf{13.6 \pm 0.7^{ab}}$	$\mathbf{16.1 \pm 1.1^{bc}}$	$\mathbf{12.2 \pm 0.8^{a}}$	$\mathbf{17.1 \pm 1.3^{c}}$
Polyunsaturates				
18:2(6)	13.8 ± 0.5^{ab}	11.5 ± 0.9^{a}	13.9 ± 0.7^{ab}	14.6 ± 1.7^{b}
18:3(3)	0.47 ± 0.06	0.61 ± 0.09	0.48 ± 0.13	0.43 ± 0.12
20:2(6)	0.12 ± 0.03^{ab}	0.34 ± 0.05^{c}	0.07 ± 0.02^{a}	0.22 ± 0.04^{b}
20:3(6)	0.30 ± 0.19	0.63 ± 0.27	0.83 ± 0.4	0.75 ± 0.28
20:4(6)	16.8 ± 0.3^{ab}	17.4 ± 0.8^{b}	16.6 ± 0.7^{ab}	14.8 ± 1.0^{a}
20:5(3)	0.05 ± 0.01	0.04 ± 0.02	0.23 ± 0.13	0.09 ± 0.05
22:4(6)	1.63 ± 0.06	1.26 ± 0.11	1.26 ± 0.24	1.26 ± 0.13
22:5(6)	0.85 ± 0.09^{ab}	0.53 ± 0.09^{a}	1.09 ± 0.21^{bc}	1.45 ± 0.31^{c}
22:5(3)	0.78 ± 0.05^{a}	1.10 ± 0.07^{b}	0.71 ± 0.07^{a}	1.11 ± 0.08^{b}
22:6(3)	1.14 ± 0.13^{ab}	1.45 ± 0.09^{bc}	0.85 ± 0.09^{a}	1.82 ± 0.31^{c}
Total CLA	0.33 ± 0.04^{a}	0.71 ± 0.14^{b}	0.30 ± 0.09^{a}	$0.33 \pm .0.09^{a}$
Total Polyunsaturates	$\mathbf{35.9 \pm 0.9}$	$\mathbf{36.7 \pm 0.8}$	$\mathbf{36.4 \pm 1.8}$	$\mathbf{37.8 \pm 1.3}$

Table 3.9. Fatty Acid Profile of the Phosphatidylcholine Fraction of Splenocytes Stimulated for 48 Hours with Concanavalin A as Determined by Gas Liquid Chromatography. *Values represent mean ± SEM with n≥7 for each dietary treatment group. Different superscripts indicate significant differences between dietary treatments ($p<0.05$).*

There was significantly more CLA incorporated into the PC fraction of splenocytes from animals fed CLA in a low P/S diet compared to the other dietary treatments. Surprisingly, the high P/S, CLA-supplemented animals demonstrated similar levels of CLA incorporation into PC as animals not fed CLA.

Within the low P/S diets, CLA supplementation reduced linoleic acid and increased arachidonic acid content as a percentage of total fatty acids. No significant differences were noted in the low P/S diets in the fatty acids involved in the ω-3 metabolic pathway (elongation and desaturation of 18:3(3)). Additionally, no differences were noted in the polyunsaturated fatty acid profiles between the two high P/S diet treatments.

Fatty Acid	Diet			
	P/S=1.0 + CLA	*P/S=0.2 + CLA*	*P/S=1.0 - CLA*	*P/S=0.2 - CLA*
Saturates				
16:0	11.5 ± 0.9^{a}	13.4 ± 1.1^{ab}	12.0 ± 1.5^{a}	16.0 ± 1.1^{b}
17:0	0.26 ± 0.08	0.16 ± 0.05	0.14 ± 0.03	0.30 ± 0.07
18:0	23.3 ± 1.8^{a}	22.8 ± 1.3^{a}	28.3 ± 0.5^{b}	20.8 ± 1.8^{a}
Total Saturates	**34.1 ± 0.8^{a}**	**36.2 ± 0.7^{a}**	**40.4 ± 1.7^{b}**	**35.5 ± 1.0^{a}**
Monounsaturates				
16:1	0.60 ± 0.11^{ab}	0.64 ± 0.19^{ab}	0.31 ± 0.07^{a}	0.81 ± 0.15^{b}
18:1	8.55 ± 0.68^{ab}	10.7 ± 1.4^{bc}	6.34 ± 0.78^{a}	11.4 ± 1.0^{c}
20:1	0.32 ± 0.09^{a}	0.19 ± 0.04^{a}	0.56 ± 0.13^{b}	0.15 ± 0.05^{a}
Total Monounsaturates	**9.41 ± 0.77^{ab}**	**13.3 ± 1.7^{bc}**	**7.32 ± 0.88^{a}**	**12.3 ± 1.0^{c}**
Polyunsaturates				
18:2(6)	14.0 ± 1.3	10.2 ± 1.9	9.40 ± 1.06	14.5 ± 2.8
18:3(3)	0.26 ± 0.03^{b}	0.34 ± 0.08^{b}	0.07 ± 0.03^{a}	0.03 ± 0.02^{a}
20:2(6)	0.13 ± 0.04^{a}	0.26 ± 0.06^{ab}	0.13 ± 0.04^{a}	0.32 ± 0.06^{b}
20:3(6)	0.13 ± 0.05^{ab}	0.26 ± 0.13^{ab}	0.36 ± 0.14^{b}	0.05 ± 0.03^{a}
20:4(6)	26.1 ± 1.6^{b}	24.8 ± 2.6^{b}	30.1 ± 2.6^{b}	19.0 ± 2.8^{a}
20:5(3)	0.25 ± 0.10^{ab}	0.06 ± 0.02^{a}	0.26 ± 0.10^{ab}	0.49 ± 0.18^{b}
22:4(6)	5.38 ± 0.71^{b}	2.59 ± 0.62^{a}	3.76 ± 1.25^{ab}	3.18 ± 0.53^{a}
22:5(6)	2.60 ± 0.26^{b}	1.28 ± 0.32^{a}	1.08 ± 0.33^{a}	1.05 ± 0.23^{a}
22:5(3)	1.05 ± 0.28	1.84 ± 0.36	1.77 ± 0.25	1.10 ± 0.31
22:6(3)	4.25 ± 0.73	4.04 ± 0.37	3.75 ± 1.06	3.28 ± 0.65
Total CLA	0.32 ± 0.05^{a}	0.54 ± 0.05^{b}	0.18 ± 0.05^{a}	0.21 ± 0.05^{a}
Total Polyunsaturates	**54.5 ± 2.0^{a}**	**46.8 ± 2.7^{b}**	**51.0 ± 2.3^{ab}**	**45.9 ± 2.0^{b}**

Table 3.10. Fatty Acid Profile of the Phosphatidylethanolamine Fraction of Splenocytes Stimulated for 48 Hours with Concanavalin A as Determined by Gas Liquid Chromatography. *Values represent mean ± SEM with $n \geq 6$ for each dietary treatment group. Different superscripts indicate significant differences between dietary treatments ($p<0.05$).*

Similar to the PC fatty acid profile (***Table 3.9***), CLA demonstrated significantly higher incorporation into the PE fraction of splenocytes from animals fed CLA in a low P/S diet compared to the other dietary treatments. Again, the high P/S, CLA-supplemented animals demonstrated similar levels of CLA to the animals not fed CLA.

Within the low P/S diets, CLA supplementation did not reduce linoleic acid content. Similar to findings for the PC fraction (***Table 3.9***) and adipose TG (***Table 3.7***), arachidonic acid (20:4(6)) was increased as a percentage of total fatty acids. Also, for low P/S diets, the percentage of LNA (18:3(3)) was increased with CLA supplementation, however EPA was decreased.

In the high P/S diets, there were increases in the percentage of LNA with CLA supplementation.

N) Body Composition

The outlier values (values ± 2SD from the mean) were from female animals, so that the remaining female sample size did not provide sufficient data for statistical analysis on the female population. Therefore, the data presented are for males only as determined by a 2-way ANOVA testing P/S ratio and CLA supplementation. Data are presented on a whole body (water included) basis. The rationale for this is explained in ***Appendix B***.

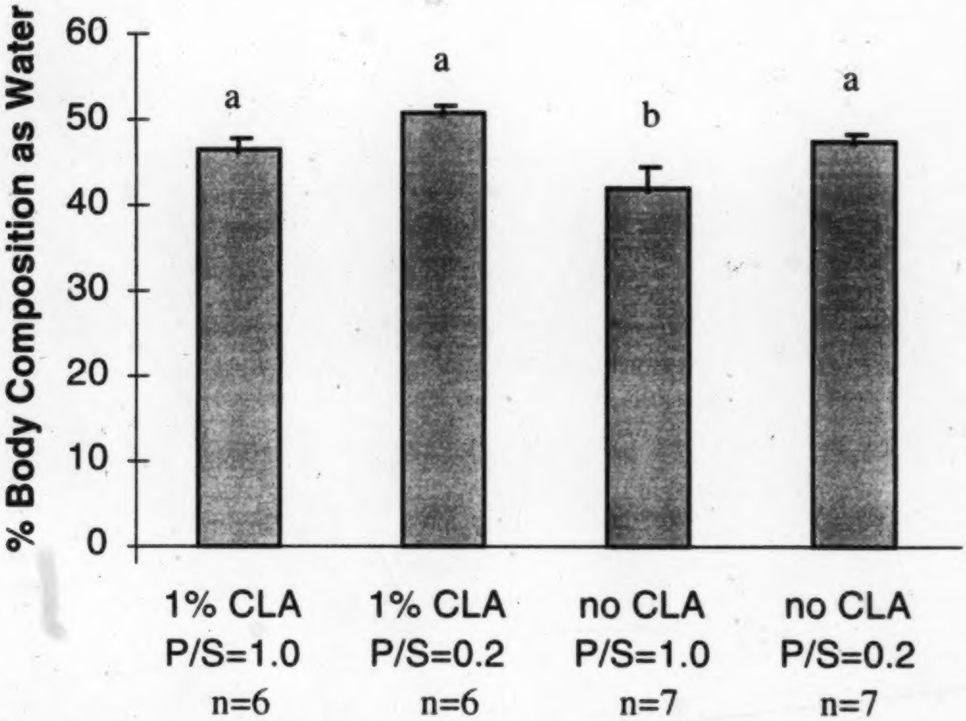


Figure 3.19. Percentage of Total Body Composition as Water. *Bars represent mean ± SEM. Bars that do not share the same letter are significantly different (p=0.05).*

Animals fed the high P/S diet without CLA had a significantly lower proportion of water (10% less) as a percent of total body weight (p=0.05) compared with animals fed the CLA-supplemented high P/S diet.

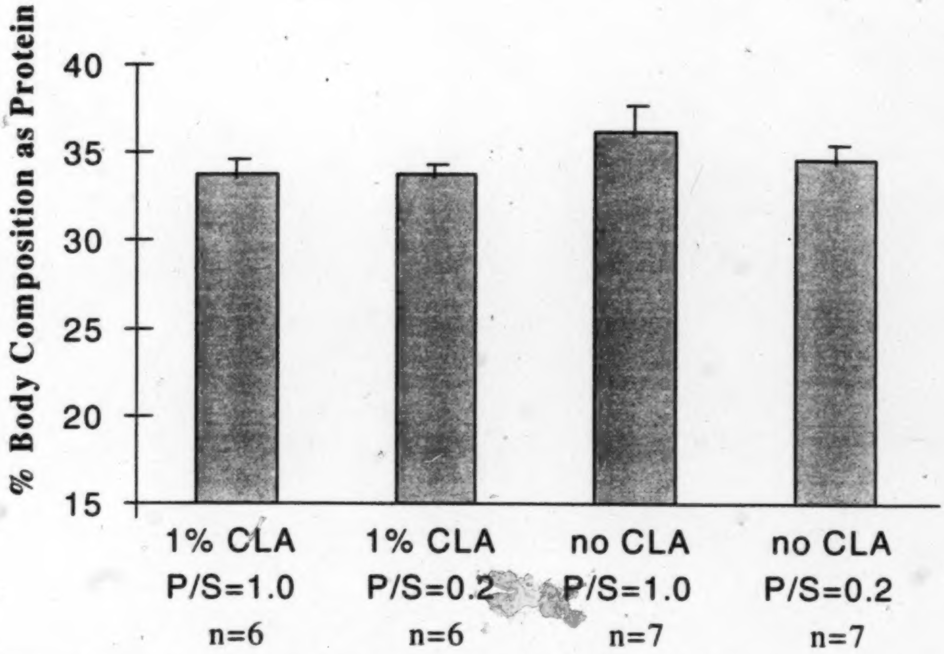


Figure 3.20. Percentage of Total Body Composition as Protein. *Bars represent mean ± SEM.*

There were no significant differences among treatment groups in the percentage of total body composition as protein.

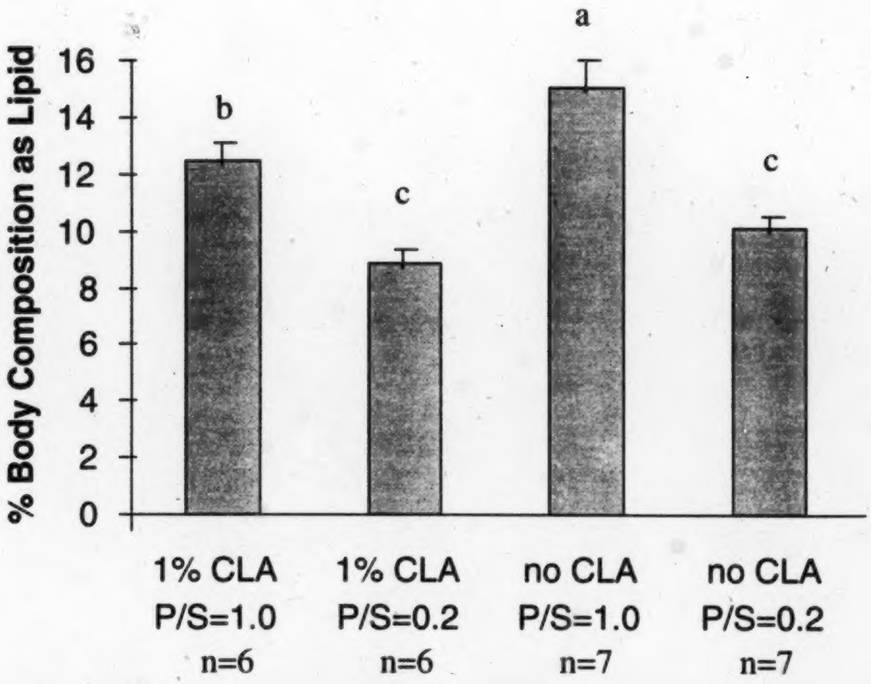


Figure 3.21. Percentage of Total Body Composition as Lipid. *Bars represent mean ± SEM. Bars that do not share the same letter are significantly different ($p<0.05$).*

Rats fed a high P/S diet demonstrated a greater percentage (45% more) of total body composition as lipid compared to rats fed a low P/S diet ($p=0.0001$). For animals fed the high P/S diet, CLA supplementation resulted in a significantly lower percentage (17% less) of total body composition as lipid ($p=0.01$).

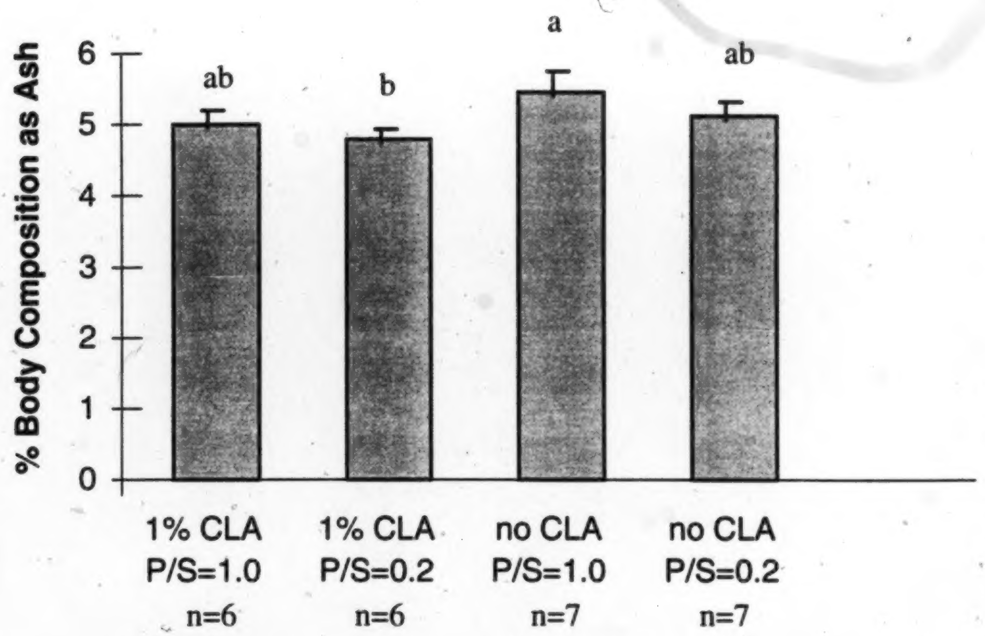


Figure 3.22. Percentage of Total Body Composition as Ash. *Bars represent mean ± SEM. Bars that do not share the same letter are significantly different (p<0.05).*

P/S ratio and CLA supplementation were not found to affect percent body composition as ash. There was, however, a significant increase (14% more) in body ash in the high P/S diet animals not fed CLA compared to the low P/S diet animals that were supplemented with CLA (p=0.05).

IV. DISCUSSION

A) Food Intake and Body Weight

Intake data (***Figure 3.4***) indicate that all treatment groups ate similar amounts removing this as a possible confounding influence on immune function and lipid and body composition. Previous rodent dietary studies examining dietary supplementation with CLA concur with this finding (Chin *et al.*, 1994A; Belury and Kempa-Steczko, 1997; Park *et al.*, 1997; Wong *et al.*, 1997).

Because the diet model used in the present study was of a higher fat content than standard laboratory rat chow, it could be argued that the differences in fat content might change the growth curve of the rat. However, several studies indicate that increasing fat content does not significantly alter growth (reviewed by NRC, 1978). In addition, body weights of the animals in the current study at 28 days were similar to the average body weight of a Fischer 344 rat at 28 days as determined by NRC (1978). It might also be argued that the diets in the current study result in more "drastic" physiological effects at weaning due to a greater dietary transition than if the animals were weaned to a chow diet. However, the experimental diets were more similar to dam's milk in lipid composition (higher in fat and contained more saturated fat) than laboratory chow resulting in less of a dietary transition at weaning. The use of this dietary model was crucial in being able to examine the effects of CLA and P/S ratio in the context of what human dietary patterns are. The use of chow was therefore not acceptable as both fat level and composition are significantly different from North American dietary patterns and would prevent extrapolation from the rat model to the human condition.

There were differences in body weight (***Figure 3.3***). The data indicate that feeding a high P/S diet resulted in a significant increase in body weight compared to low P/S diets after receiving dietary treatment for as little as five days ($p<0.02$). These results are likely due to differing absorption of fat rather than different lipid metabolism and storage. According to Lien *et al.* (1997), saturated fats are more poorly absorbed in the intestine than polyunsaturated fats. This is particularly true when saturated fats are located in the sn-1 or sn-3 position of a triglyceride (Lien *et al.*, 1997), as is the case for stearine, the source of saturated fat used in the diets in this study. Therefore, rats fed the low P/S diets may have been absorbing less lipid, resulting in less energy intake and a lower body mass. Other studies indicate that P/S ratio can alter body weight. In a study by Field and Clandinin (1990A), diets containing higher levels of dietary fat (20% w/w), which approximate the proportion of energy in human diets, were fed to weanling Sprague Dawley rats. Those fed a high P/S diet (P/S=2.0) weighed significantly more than those fed a low P/S diet (P/S=0.2). An effect of P/S ratio on body weight was not noted when a lower level of fat (10% w/w) was used. This was true in both controls and diabetic animals. Field *et al.* (1990B) found similar results in a separate experiment, however when older animals were used and fed for a shorter period of time (2 weeks), the effects of dietary P/S ratio on body weight were not seen. These results, together, suggest that several factors may be involved in the modulation of body weight by P/S ratio. These include the level of dietary lipid, the length of feeding, and the age of the animal.

The data obtained for epididymal fat pad weight (***Figure 3.6***) can be similarly explained. Animals fed a high P/S diet had greater fat storage due to the fact that they absorbed more energy. It is interesting to note, however, that the epididymal fat pad weights of the rats fed the two high P/S diets were different (p=0.03). The CLA-supplemented high P/S diet group had lower epididymal fat pad/body weight than did the non-supplemented high P/S diet group. As food intake did not differ between these two high P/S groups, this suggests an effect of CLA on body fat deposition or utilization of fat. CLA supplementation did not affect epididymal fat pad weight in animals fed low P/S diets (p=0.38) indicating that reductions in adipose storage due to CLA may be associated with diets high in PUFA.

In support of these findings, a similar effect of P/S ratio and CLA on body fat was seen when percent body composition as lipid was analyzed on a whole body weight basis (***Figure 3.19***). Animals fed a high P/S diet had a higher proportion of carcass fat compared to animals fed a low P/S diet (45% higher, p=0.0001). As well, CLA had no effect on body lipid in rats fed low P/S diets. There were significant differences in body lipid content for animals fed high P/S diets. CLA supplementation reduced the percent body composition as lipid by 17% compared to animals not receiving CLA. (p=0.01). This trend is similar, albeit of lower magnitude, to Park and colleagues (1997) who found a 57% decrease in body lipid and an approximate 10% increase in lean body mass in male mice fed standard laboratory chow supplemented with 0.5% (w/w) CLA for 4 weeks. These animals received 5.0% (w/w) corn oil as their dietary fat source so that they also were receiving a high P/S diet. In concurrence with this, Dugan *et al.* (1997) found that 2% (w/w) CLA supplementation versus 2% (w/w) sunflower oil in a cerealbased diet (high P/S ratio) resulted in significantly less subcutaneous fat (-6.8%) and increased lean body mass (+2.3%) in pigs. These diets were begun when animals averaged 61.5 kg and continued to a slaughter weight of approximately 106 kg. Comparison between the present study and previous work is difficult due to the variation in species studied, the diet into which CLA was supplemented, the amount of CLA supplemented, and length of supplementation. Also, because of the wide variations between the studies in the magnitude of change in body lipid, it is difficult to determine the physiological relevance of this change. However, in our study, a 17% reduction in body lipid of healthy growing rats was seen. If one attempts to extrapolate this finding to adult males with an average body fat composition of 15% body lipid, CLA supplementation would lower body lipid to 12.5%, a relatively small change.

Therefore, in the current study, CLA reduced adipose stores (i.e. epididymal fat pads) and overall body lipid content in animals fed high P/S diets. The similar results obtained by Park *et al.* (1997) and Dugan *et al.* (1997) utilized diets which were lower in fat content. Our data suggest that CLA alters the percent body composition as lipid when animals are fed high P/S diets which are higher in fat content and representative of human dietary recommendations. It is not known why CLA would only modulate body composition in higher P/S diets. A modulatory mechanism was proposed by Pariza and colleagues (1997) who demonstrated that epididymal adipocytes from rats fed 0.5% CLA demonstrated enhanced norepinephrine-induced lipolysis and hormone sensitive lipase activity. The lack of change in body composition with low P/S diets may be due to the reduction in lipolytic activity and β-adrenergic receptor binding seen with beef tallow diets (Matsuo *et al.*, 1995). As the low P/S diets in our study utilized stearine (beef tallow) as the source of saturated fat, the beef tallow may have counterbalanced the lipolytic effects of CLA.

Changes in body composition were not noted in percent of body protein (***Figure 3. 21***). The findings of previous studies (Park *et al.*, 1997; Dugan *et al.*, 1997) do not concur with these results. Several factors including differences in diet composition, sample size, age of experimental animals and length of dietary treatment may all have contributed to the lack of effect seen on body protein. Such differences in experimental design make comparison difficult between our study and previous work. Whereas other researchers were examining body compositional changes with respect to feed efficiency and animal weight gain for agricultural purposes, our study was examining body composition in a dietary context more relevant to that consumed by and recommended for humans.

B) CLA and Immune Function

i) Mitogen-Induced Proliferation

An immunological parameter that differed with CLA supplementation was mitogen-induced proliferation. Previous studies have demonstrated that several individual fatty acids inhibit splenocyte T cell proliferation. These include dietary oleic acid (Jeffery *et al.*, 1997), and α-linolenic acid (Jeffery *et al.*, 1996), as well as *in vitro* studies with linoleic, γ-linolenic, dihomo-γ-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acid (Calder, 1995; Tsang *et al.*, 1977). In addition, the effects of saturated fatty acids have been found to be less inhibitory than those of unsaturated fatty acids (Calder, 1991). Although individual fatty acids have been studied, the effects of P/S ratio had not been examined to this point.

Two mitogens were used in our experiment. Con A is a T-cell mitogen which induces proliferation through actions at the membrane (Calder, 1995). The combination of PMA + Iono induce proliferation via intracellular sites (i.e. beyond the membrane). Both P/S ratio ($p=0.01$) and CLA ($p=0.05$) were found to alter Con A-induced proliferation (***Figure 3.10***). The data indicate that the low P/S diets resulted in greater immunoproliferation than the high P/S diets. This seems logical since the low P/S diets contain less linoleic acid- a polyunsaturated fat shown to reduce proliferation of immune cells (Calder, 1995). Within the low P/S diets, CLA supplementation enhanced Con A-induced proliferation ($p=0.04$). This could be due to membrane fatty acid composition change, particularly the reduction in LA incorporation, which will be discussed in detail shortly. A metabolite of LA, PGE_2, has been shown to be associated with reduced lymphocyte proliferation (Goodwin *et al.*, 1977). In addition to enhancing the degree of proliferation, the low P/S diet supplemented with CLA also demonstrated a more rapid proliferative response (***Figure 3.9***). These results together indicate that supplementing CLA into a diet of similar composition to what many North Americans consume enhances both the speed and degree of T cell response. Such enhanced immune function might lead to more rapid elimination of immune stimuli (i.e. tumor cell antigens) at an earlier stage of tumor development which would, in turn, reduce tumorigenesis.

When PMA + Iono were used, P/S ratio did not have a significant effect on proliferation. This may be due to the fact that the immunoproliferative mechanism of PMA + Iono bypasses the membrane receptor which could be the site at which P/S ratio affects proliferation. CLA supplementation did, however, enhance splenocyte proliferation due to PMA + Iono (p=0.05). There was no difference between the high P/S diets. However, within the low P/S diets, CLA supplementation enhanced proliferation relative to the non-supplemented diet (p<0.03). These trends are similar to those seen with Con A. That CLA supplementation enhances proliferation due to PMA + Iono suggests that CLA may enhance proliferation at post-membrane sites.

Previous studies concur that CLA enhances immunoproliferation. *In vitro* studies by Chew and colleagues (1997) indicated that physiological levels of CLA stimulated mitogen-induced proliferation of porcine lymphocytes. Dietary studies by Wong *et al.* (1997) revealed an increase in PHA (another T-cell mitogen)-induced lymphocyte proliferation when 0.3-0.9% w/w CLA was supplemented into the diet. Miller *et al.* (1994) demonstrated that 0.5% w/w CLA in the diet enhanced splenocyte blastogenesis due to endotoxin injection. In contrast to our experiment, these dietary studies were carried out using laboratory chow diets. Laboratory chow is low in fat but high in polyunsaturated fatty acids. Also in contrast, our results did not demonstrate that CLA supplementation enhanced proliferation in higher P/S ratio diets such as a laboratory chow would provide. The lack of an effect that we noted with high P/S diets may have been due to the higher fat content of our diets. Kelley *et al.* (1992) suggested that high fat diets in humans may suppress immunoproliferation. At 15% w/w, our diets were formulated at a higher lipid level than laboratory chow. While the enhancing effects of CLA supplementation in low P/S diets may outweigh the immunosuppressive effects of a higher lipid content, this may not be true of high P/S diets. Nonetheless, our use of a higher lipid content is essential to translate results to the human diet. Our results indicate that, at the current recommended level of lipid (30% w/w), CLA most effectively elicits an immunoproliferative response in a diet with more saturated fat, rather than more polyunsaturated fat as currently recommended.

ii) Characterization of Immune Cells in Spleen

Our study attempted to examine, for the first time, the effects of CLA and P/S ratio on immune cell subpopulations, both freshly isolated cells as well as splenocytes cultured for 48 hours with mitogen stimulation. Phenotypic analysis of freshly isolated splenocytes indicated no effects of diet P/S or CLA supplementation on the proportion of T cells (ox19+), T helper cells (w3/25+), T suppressor cells (ox8+), B cells (ox12+), macrophages (ox42+) or NK cells (3.2.3+). Little research to date has examined the effect of diet on immune populations following stimulation. Robinson and Field (1998) found an enhancing effect of high dietary ω-3 fatty acids on immune cell activation as determined by expression of the IL-2 and transferrin receptors. These effects were noted in diets where P/S ratio=0.9. In the present study, consistent diet effects were noted on immune subpopulations of splenocytes cultured for 48 hours with Con A. Animals fed the low P/S diet with CLA supplementation exhibited a higher proportion of several immune subpopulations relative to animals fed the non-supplemented low P/S diet. The lack of effect of CLA in the high P/S diets may have been due to the immunosuppressive effects of PUFA on various subpopulations including those expressing the transferrin receptor (ox26+, Calder and Newsholme, 1992). Therefore, all subsequent discussion on phenotypic analysis will be comparing the two low P/S dietary groups only. CLA supplementation increased the proportion of cells expressing the transferrin receptor (ox26+, p=0.05) and IL-2 receptor (ox39+, p=0.06). These receptors are both essential for proliferation of T cells (Neckers and Cossman, 1983) and their expression is coupled with cell proliferation (Hirsch *et al.*, 1996). CLA supplementation enhanced expression of both these receptors on the total T cell, T helper cell, T suppressor cell, B cell and macrophage populations. The enhanced expression of these markers indicate that a larger proportion of immune cells are activated and proliferating. This may suggest that, in the presence of a physiological stimulus (i.e. a tumor antigen), a larger proportion of immune cells would become activated and involved in mounting an immune response against the antigen. This could lead to tumor cell destruction and a reduction in tumor development. The increased activation does not appear to be due to differences in the CD4+:CD8+ ratio (w/25:ox8; ***Table 3.4***) or IL-2 production (***Figure 3.12***). No differences in the stimulated populations of w3/25+ (CD4+) or ox8+ (CD8+) or in the ratio between them were noted among the diet treatments. Since CD4+ cells are a major producer of IL-2, and there were no differences in this population due to diet, this may explain the lack of an effect on IL-2 production. Therefore, the increased proliferation seen with the low P/S diets and with CLA supplementation in a low P/S diet may be due to other mechanisms such as membrane composition changes or alterations in second messenger pathways.

Our results indicated that P/S ratio did not modulate any of the immune populations studied in either freshly isolated or cultured cells. Several subpopulations demonstrated a P/S*CLA interaction however (***Figures 3.5*** and ***3.6***). CLA supplementation consistently enhanced expression of activation markers when added to a low P/S diet.

iii) Natural Killer Cell Activity

NK cells are a component of the innate immune system which are involved in the detection and lysis of bacterially and virally infected cells. As well, NK cells can lyse altered self cells such as tumor cells (Hanna, 1985). Therefore, a change in the lytic ability of NK cells may provide a mechanism of reduced tumor development. The Natural Killer Cytotoxicity assay (***Figure 3.7***) indicated that all treatment groups had similar cytotoxic abilities at all ratios studied. Phenotypic data indicated a lower NK population in the high P/S non-CLA supplemented group (***Table 3.2)***, however when lysis was expressed in lytic units (the number of NK cells required to cause 20% lysis of target cells), there were no significant differences. It would appear from this data that neither dietary fat composition nor CLA supplementation in diets that represent current human dietary patterns alter NK lytic ability. This study is the first to examine NK cytotoxicity, however other CLA studies have examined general immune cytotoxicity as well as macrophage cytotoxicity. Wong *et al.* (1997) saw no effect on immune cytotoxicity *in vivo* when mice were fed 0.3-0.9% CLA for 3 or 6 weeks. In contrast, work by Chew *et al.* (1997) demonstrated enhanced macrophage cytotoxicity *in vitro* using physiological concentrations of CLA. Although Chew and colleagues observed effects on cytotoxicity, they did not attempt to elucidate the mechanism (i.e. increase in macrophage population, enhanced production of lytic compounds, etc.).

Previous studies regarding lipid composition and NK cytotoxicity suggest that a relationship exists. Robinson and Field (1998) demonstrated that 3.3% (w/w) fish oil in a diet containing 20% (w/w) lipid increased NK cytotoxicity in sedentary rats compared to rats not fed fish oil. In contrast, Jeffery *et al.* (1996, 1997) determined that ω-3 fatty acids reduced NK cytotoxicity and that palmitic acid enhanced cytotoxicity in diets formulated to contain similar lipid levels to that consumed by North Americans, some of which also represented North American dietary lipid composition (i.e. P/S ratio= 0.28 - 5.56). In agreement with our results, Jeffery and colleagues did not find a significant correlation between NK activity and dietary P/S ratio. The literature to this point has focussed largely on the effects of specific fatty acids on NK cytotoxicity (Yaqoob *et al.*, 1998) rather than on fatty acid composition and P/S ratios. The results of our study indicate that P/S ratios which are similar to that consumed and recommended for the human population do not alter NK cytotoxic activity nor does CLA exert any effects on NK cytotoxicity within these physiologically relevant ratios.

iv) Nitric Oxide production

The NO data (***Figures 3.13*** and ***3.14***) concurred with our NK cytotoxicity results in that there were no significant differences in NO production between the different diet treatment groups for cultured splenocytes that had been stimulated with Concanavalin A as well as those which were not stimulated. NO is a compound produced by NK cells and macrophages to lyse their targets (Filep *et al.*, 1996). A difference in killing ability might, therefore, be accompanied by a difference in production of this lytic substance.

Individual fatty acids, such as EPA and DHA, have been shown to have a suppressive effect on production of NO (Khair-el-Din *et al.*, 1996) whereas LA, oleic acid and stearic acid have not (Ohata *et al.*, 1997). However, no research published to date has examined the effect of CLA or P/S ratio on NO production. Therefore, the current study provided novel information demonstrating that CLA supplementation and modulation of dietary lipid composition within human dietary levels (current versus recommended lipid composition) do not alter NO production. This is consistent with the results on NK activity.

v) IL-2 Production

Despite changes in splenocyte proliferation, no significant differences were seen in IL-2 production (***Figure 3.12***)- a cytokine involved in initiating and propagating proliferation of T cells. In contrast, Wong and colleagues (1997) found that feeding mice 0.3-0.9% CLA for three to six weeks increased production of IL-2. Again, perhaps these differing results are due to the levels of lipid used. Yaqoob and Calder (1995) demonstrated that feeding a 2.5% w/w lipid diet to mice reduced IL-2 production by splenocytes relative to some diets of higher lipid content. Similarly, Venkatraman and colleagues (1997) demonstrated that increasing dietary fat can increase IL-2 production by peripheral blood mononuclear cells in humans. Perhaps the higher fat levels fed in our diets may have enhanced IL-2 production in a similar manner, thus masking any stimulatory effects of CLA which might be seen with a lower lipid content. It is also possible that 48 hours was not the most appropriate time at which to examine IL-2 production. Because Con A leads to maximal proliferation at 90 hours (***Figure 3.10***), an earlier time point was chosen at which to study IL-2. The time point chosen may have been prior to or following peak IL-2 production which may have masked any differences due to dietary treatment. However, because one treatment group (low P/S, CLA added; ***Figure 3.9***) already demonstrated heightened immunoproliferation at 42 hours, one would guess that higher IL-2 production for that group would have also been detected, if enhanced IL-2 secretion were the immunoproliferative mechanism. Although no differences in IL-2 production were noted among dietary treatments, our use of diets with a higher lipid content was crucial in understanding the potential physiological effects of CLA for humans in the context of human dietary lipid patterns.

Other individual fatty acids have been found to alter IL-2 production. When added at 20% w/w of diet, olive oil and safflower oil both enhanced IL-2 (Yaqoob and Calder, 1995). Jolly *et al.* (1997) determined that adding 1% w/w EPA or DHA to diets containing 3-4% w/w lipid resulted in inhibition of IL-2 production. These studies indicate that fatty acid composition can modulate production of this cytokine, however the physiological relevance of such dietary supplementation is questionable. The effects of human dietary lipid patterns, particularly lipid composition, on cytokine production have not been specifically examined. Our data indicate that diets designed similar in fat content and PUFA composition to human dietary lipid patterns or recommended lipid patterns do not result in modulation of IL-2 production, nor does CLA alter this effect.

vi) IFN-γ Production

Macrophages exert potent tumoricidal activity when activated by various stimuli including IFN-γ that is produced by Th1 cells (Somers *et al.*, 1989). Changes in the production of this cytokine, would suggest more of a Th1-type response and might modulate macrophage tumoricidal activity. Previous studies indicate that these two factors may be altered by dietary fatty acids. Specifically, Erickson and Hubbard (1994) found that mice fed menhaden fish oil had reduced macrophage responsiveness to IFN-γ compared to mice fed safflower oil. This group did not measure endogenous IFN-γ production however. Fritsche *et al.* (1997) fed mice diets containing 20% (w/w) lipid composed of soybean oil, lard or menhaden and corn oil (17:3 w/w). Mice were then injected with *Listeria monocytogenes*. The researchers found that animals fed the fish oil diet had signficantly higher serum IFN-γ compared to the other two treatment groups. Such elevated levels might enhance the ability of the immune system, particularly macrophages, to eliminate tumor cells.

No previous studies have examined the effects of CLA on IFN-γ production. Therefore, the current study provided novel information and suggested that there was no effect of either P/S ratio or CLA supplementation on production of IFN-γ in unstimulated or stimulated splenocytes. However, the animals used in the current study were not immune-challenged (i.e. no tumor burden). Future studies may wish to examine IFN-γ production in animals with a tumor burden. Differences in production of this and other cytokines examined might be noted under conditions of immune challenge, such as those noted by Fritsche and colleagues (1997).

vii) TNF-α Production

TNF-α, also known as cachectin, was originally discovered as a factor in serum which was capable of causing necrosis of certain tumor cells (Carswell *et al.*, 1975). A major site of TNF-α production is the macrophage (Hubbard and Erickson, 1996). TNF-α is also produced by Th1 and Th2 CD4+ cells. Previous studies examining TNF-α production indicate that levels may be altered by dietary fatty acids. For example, Erickson and Hubbard (1994) found that macrophages from mice fed menhaden fish oil produced significantly more TNF-α than macrophages from mice fed safflower oil after 24 hours stimulation with LPS. Similar effects on TNF-α production have been demonstrated *in vitro* by Tappia and colleagues (1995). This group incubated rat peritoneal macrophages with 100 μM of various fatty acids and found increased TNF-α production in macrophages treated with DHA or EPA. Similar enhancing effects of ω-3 fatty acids have been noted in malnourished cancer patients by Gogos and colleagues (1998).

No previous studies have examined the effects of CLA on TNF-α production. Therefore, the current study provided novel information and suggested that despite a difference in proliferative response to P/S ratio or CLA supplementation there was no difference in this macrophage and Th1 derived cytokine in either unstimulated or stimulated splenocytes. However, the animals used in the current study were not immune-challenged (i.e. no tumor burden). Future studies may wish to examine TNF-α production in animals with a tumor burden. Differences in production of this cytokine might be noted under conditions of immune challenge.

In examining all cytokines, it can be seen that there were no diet differences in IL-2, IFN-γ, or TNF-α which are all Th1 cytokines. Therefore, the increase in proliferation which was seen may have been mediated by changes in Th2 cytokines, which were not examined.

C) CLA Incorporation into Tissues

i) Adipose Triglycerides

CLA was supplemented in the diet at 1% (w/w) or approximately 6% of lipid. Incorporation of CLA was noted in both adipose TG (***Table 3.7***) and immune cell PL (***Tables 3.9 and 3.10***). Previous studies have found CLA in these lipid fractions (Chin *et al.*, 1994; Ip *et al.*, 1996). Also in agreement with our findings, Chin and colleagues (1994) noted a greater relative proportion of CLA in neutral lipids (NL) compared to PL.

Our results indicated that non-supplemented animals contained only trace amounts of CLA, as would be expected. These trace amounts may be due to stearine, our saturated fat source, which is derived from beef tallow. As discussed previously, ruminant products contain CLA. The trace quantities may also have been contributed to by endogenous synthesis of CLA by bacterial flora in the intestine (Chin *et al.*, 1994).

Interestingly, the two groups that were supplemented with 1% CLA demonstrated different incorporation of CLA into their adipose TG. The low P/S diet group had 5.65±0.25% of total fatty acids as CLA which is proportional to dietary CLA levels (surprisingly similar to the total proportion in the diet). The high P/S group, however, had only 2.86±0.26% of total fatty acids in adipose tissue as CLA. The lower incorporation seen with the high P/S diet is noteworthy when one considers that the animals fed high P/S diets had higher body and epididymal fat pad weights suggesting a more efficient absorption of fat as previously discussed. Less incorporation of CLA with a high P/S ratio (despite a better absorption of dietary fat) suggests competition with other fatty acids, particularly LA, a geometric and positional isomer of CLA.

Previous research is inconclusive as to whether this competition exists. Kramer and co-workers (1998) found that the distribution of CLA isomers in inner back and omental fat was similar to diet levels. This group examined total lipids rather than a single fraction, however, and CLA was supplemented into a standard chow diet unlike the composition used in our study. Liew *et al.* (1995) did not see modulations in colon or liver total fat LA, LNA or AA when CLA was delivered by gavage at a level equivalent to 0.5% (w/w)/day. Again, these animals were receiving chow diets and CLA was delivered in an olive oil gavage making comparisons to our results difficult. In examining the mammary gland, Ip *et al.* (1996) found that neither the efficacy of tumor suppression nor the incorporation of CLA into neutral lipids was affected by the intake of LA. This group also determined that CLA supplementation did not appear to displace LA or AA in mammary tissue. Although these investigators attempted to simulate North American dietary lipid patterns, it is difficult to compare their findings in mammary tissue to our findings in epididymal fat pads as there are variations in lipid metabolism and storage with different organs and tissues (Kramer *et al,* 1998; Christensen *et al.*, 1998; Liu and Longmore, 1997).

In contrast to the studies which indicated that CLA did not selectively displace other FA, Cook and colleagues (1993) found a decrease in abdominal fat pad AA with CLA supplementation in a semi-purified chow diet. Because these animals were immune-challenged with PHA, it is likely that their lipid metabolism would differ from our healthy animals (Memon *et al.,* 1998). Finally, Belury and Kempa-Steczko (1997) determined that CLA incorporated into the neutral lipids of liver by displacing LA. They also saw an increase in oleic acid and a decrease in AA.

Therefore, the literature is difficult to interpret due to the variations that exist between the studies. These include the tissue being studied, the experimental diet administered, the means of administering CLA, the health of the animals, and the lipid fraction analyzed. Our data indicate that, in healthy animals, feeding CLA in diets containing a lipid composition similar to current North American patterns and at the current recommended lipid level results in greater incorporation of CLA in adipose TG (***Table 3.7***) and splenocyte PC (***Table 3.9***) and PE (***Table 3.10***) than when it is supplemented into diets containing more PUFA as currently recommended. The reasons for this difference are not known but may be due to competition between CLA and other fatty acids such as LA. Such competition would result in altered FA metabolism and storage with different dietary lipid composition. Alterations in metabolism have been noted with other fatty acids, including the competition between LA and LNA for elongation and desaturation enzymes (Brenner and Peluffo, 1966).

Dietary CLA was comprised of several isomers, however two isomers accounted for >90% of total dietary CLA. These two major isomers have been identified as the *c*9*t*11 and *t*10*c*12 isoforms (Ma *et al.*, in press B) and were present in the diet in a ratio of 1 (see ***Appendix B***). Adipose tissue distribution (***Figure 3.15***) showed that these two isomers still accounted for >90% of total CLA (***Figure 3.8***) however the ratio had increased suggesting a biological preference for the incorporation of the *c*9*t*11 isomer. In support of this, it is interesting to note that this isomer is the isomer found in beef and dairy products (Ma *et al.*, in press A) and that it is postulated to be biologically active. Results of Sehat *et al.* (1998) concurred with this in that they also saw a bioconcentration of *c*9,*t*11 in total liver lipids relative to dietary levels.

Further evidence of competition between LA and CLA was determined through examining the polyunsaturated fatty acid composition of adipose TG (***Table 3.7***) as simplified below in ***Table 3.11***.

	Diet		*Adipose TG*	
	+ CLA	- CLA	+ CLA	- CLA
LA	8.9	14.3	11.8 ± 0.4^a	19.8 ± 0.6^b
LNA	1.0	1.0	0.81 ± 0.05^a	0.83 ± 0.04^a
CLA	7.1	0.2	5.65 ± 0.25^b	0.50 ± 0.09^a
AA	---	---	0.15 ± 0.01^b	0.02 ± 0.01^a
Total PUFA	**17**	**16**	$\mathbf{18.4 \pm 0.7^a}$	$\mathbf{21.2 \pm 0.5^b}$

Table 3.11. Polyunsaturated Fatty Acid Composition of Diet and Adipose Triglycerides for Animals fed Low P/S Diets as Determined by Gas Liquid Chromatography. *Values represent mean ± SEM with n≥5 for each dietary treatment group. Different superscripts indicate significant differences between CLA- supplemented and non-supplemented animals (p<0.05).*

Among rats fed low P/S diets, those receiving CLA had a lower level of total PUFA in adipose TG compared to those not receiving CLA, despite similar dietary

PUFA levels. Fatty acid analysis also indicated that these animals had lower adipose TG levels of LA, however it could not be assumed on this basis that CLA was displacing LA. In preparing the diets, CLA replaced LA to maintain constant total PUFA levels, therefore dietary intake of LA was lower in the CLA-supplemented rats. However, the other fatty acids which comprised total adipose PUFA either were unchanged between the two diet groups (adipose LNA) or increased in the CLA-fed animals (adipose CLA and AA). Therefore, by deduction, the reduced total PUFA in adipose TG in rats fed CLA must have been due to a reduced deposition of LA into adipose stores. It is interesting to note that, despite the decreased LA levels, there was a significant increase in AA. This is in contrast to previous studies which have noted decreases in both LA and AA (Cook *et al.*, 1993; Belury and Kempa-Steczko, 1997). Radiolabelled tracer studies would be useful in determining whether this increase in AA is due to enhanced LA metabolism or other potential alterations in metabolism with CLA supplementation.

ii) Stimulated Splenocyte Phospholipid Fractions

Due to the small amount of lipid available for lipid analysis, it was not possible to accurately determine individual CLA isomer content in the isolated phospholipid fractions. For this reason, CLA is presented only as a total of all isomers. Of the four dietary treatments, the PC fraction from rats fed the low P/S, CLA-supplemented diet demonstrated enhanced CLA incorporation compared to the other three groups (***Table 3.9***). Similar to our findings with adipose tissue, this group also demonstrated reduced LA (p=0.05) and enhanced AA (p=0.04) incorporation compared to low P/S non-supplemented animals. The reasons for different effects on LA and AA are not known. LA is metabolized to AA and, therefore, it would be expected that a reduction in the former would lead to a reduction in the latter. However, it is also possible that CLA might interrupt the cleavage of AA by PLA_2 or its subsequent metabolism to PG or LT resulting in accumulation in the membrane. Future tracer studies with radiolabelled CLA are warranted to elucidate its metabolic fates and confirm competitive pathways.

We observed the same trend for CLA incorporation into the PE fraction (***Table***

3.10). The low P/S CLA-supplemented diet demonstrated a significant increase in the percent of total fatty acids as CLA compared to the other three diet treatments. This dietary treatment group also had an increase in the proportion of AA present, just as we observed for adipose TG and splenocyte PC. We also noted some significant changes in the level of ω-3 fatty acids in low P/S CLA-supplemented animals, suggesting that, for this PL fraction, CLA may alter this pathway as well.

Consistent effects on fatty acid composition of PC (***Table 3.9***) and PE (***Table 3.10***) between the two P/S ratios were not noted. A previous study on adipose tissue structural lipids indicated an association between increasing dietary LA and increasing PC P/S ratio (Field *et al.*, 1985). However, it is difficult to compare this study to ours as our study examined immune cell phospholipids, rather than adipose phospholipids. Additional variables such as cell culturing and, particularly cell activation (Calder *et al.*, 1994; Robinson and Field, in preparation) may have resulted in similar fatty acid composition between the two diets. This will be discussed below.

The concern might arise as to whether PL fatty acid profiles reflect dietary-induced membrane changes after immune cells have undergone a 48 hour culture period and have been exposed to mitogen. Regarding the effects of cell culture on membrane composition, Calder and colleagues (1994) found that, without mitogen stimulation, culturing rat lymphocytes for 48 hours did not alter fatty acid composition. However, in examining specific membrane lipid fractions, Robinson and Field (in preparation) found an effect of cell culture on the PC and PE fractions of rat splenocyte membranes. Specifically, decreases in LA and AA content, as well as in the sum of ω-6 fatty acids and the ω-6/ ω-3 ratio were seen.

The effects of mitogen stimulation on membrane composition are also an important factor to consider in the present study. Cell activation has been shown to alter the fatty acid composition of immune cell membranes. Calder *et al.* (1994) found that stimulating rat lymphocytes for 48 hours with Con A resulted in reduced levels of palmitic, stearic, LA, and AA and increased levels of oleic acid compared to freshly isolated lymphocytes or lymphocytes cultured for 48 hours without mitogen stimulation. Robinson and Field (in preparation) also noted some changes in fatty acid composition of PC and PE with mitogen stimulation, however levels of LA and total ω-6 fatty acids were not affected. These studies suggest that the greatest changes in levels of ω-6 fatty acids in the PC and PE lipid fractions are due to cell culturing rather than mitogen stimulation. Alterations in fatty acid composition with cell culture, particularly in LA, may explain in part why no effect of P/S ratio was seen on the level of PUFA in stimulated rat splenocytes (***Tables 3.9 and 3. 10***).

Importantly, however, Robinson and Field (in preparation) showed that animals fed a high ω-3 fatty acid diet had higher ω-3 fatty acid levels and a lower ω-6/ω-3 ratio in PC and PE in freshly isolated splenocytes as well as in splenocytes cultured for 48 hours with mitogen stimulation. Together, these results strongly suggest the ability to maintain diet-induced alterations in membrane composition through a 48 hour culture period with or without mitogen stimulation. Although we only examined stimulated splenocytes and, therefore, cannot compare our results with pre-culture profiles or unstimulated post-culture profiles, the trends we observed in the PC and PE fractions were similar to those noted in adipose TG. That is, there were increased levels of CLA in the PC and PE fractions of splenocytes from rats fed dietary CLA. Also, levels of CLA were higher in animals fed low P/S diets versus those fed high P/S diets. Because no differences were noted in total PUFA of splenocyte PC and PE, despite dietary differences, it appears that the effects on proliferation (***Figures 3.10*** and ***3.11)*** were not due to the P/S ratio or PUFA content but rather due to the CLA content.

In examining the data from this experiment as a whole, it can be seen that incorporation of CLA into adipose and immune membranes, and functional changes due to CLA were maximized in animals receiving a low P/S diet similar to what many North Americans currently consume. Our results also indicate that, in these same low P/S diets, CLA appears to displace LA in the TG and PC fractions. This may have been due to the fact that low P/S ratio diets are lower in polyunsaturated fat, specifically LA, so that competition between CLA and LA would be reduced. This, in turn, may have provided a more efficacious environment to enhance the biological effects of CLA.

Like previous studies (Innis and Clandinin, 1981; Garg *et al.*, 1988; Field *et al.*, 1989; Robinson and Field, in preparation), we also determined that dietary fat composition influenced membrane composition (see ***Tables 3.9*** and ***3.10***). However, whether such a membrane change resulted in changes in fluidity, ion permeability, enzyme or receptor function, gene expression, or second messenger pathways remains to be elucidated. Because this study strongly suggests competition between CLA and LA, it would seem logical to pursue research in this area. Some studies have examined such possibilities and determined that CLA may affect both the lipoxygenase (Cunningham *et al.*, 1997; Sugano *et al.*, 1998) and cyclooxygenase (Liu and Belury, 1997) pathways. More research into these possibilities will hopefully continue to examine altered LA metabolism, competition of these compounds for metabolic enzymes, and the production of novel bioactive metabolites from CLA.

V. SUMMARY

The results of this study indicated that dietary CLA did not alter measures of the innate immune system (NK cytotoxicity, NO production, IFN-γ, and TNF-α production). However, when CLA was fed in a low P/S diet, it did enhance the cell-mediated immune system (splenocyte proliferation and expression of activation markers) relative to the non-supplemented low P/S diet. Such effects on various arms of anti-cancer immune defense, specifically cell-mediated immune defenses, may suggest an anti-carcinogenic mechanism of CLA.

Similarly, CLA incorporation into adipose TG and immune cell PL was maximized when supplemented in a diet with a similar fat composition to current North American dietary lipid patterns (low P/S) rather than the current recommended composition (high P/S). Our data indicate that LA was decreased and AA was increased in adipose TG and immune cell PC from animals fed the low P/S CLA-supplemented diet compared to the non-supplemented low P/S diet. Enhanced splenocyte membrane and functional changes when CLA was supplemented in a low P/S diet, but not a high P/S diet, strongly suggest competition between LA and CLA.

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Chapter Four- Conjugated Linoleic Acid and Tumor Cell Adhesion

I. Introduction

Conjugated linoleic acid (CLA) refers to a series of positional and geometric isomers of LA. Although CLA is a relatively minor dietary constituent, it has recently received attention due to potent anticarcinogenic effects which have been demonstrated in animal models of skin (Pariza and Hargraves, 1985), forestomach (Ha *et al.*, 1990), colon (Liew *et al.*, 1995) and mammary cancer (Ip *et al.*, 1991). These effects have been noted in several tumor cell lines as well (Seaman *et al.*, 1992; Shultz *et al.*, 1992, 1993; Schønberg and Krokan, 1995; Yoon *et al.*, 1997; Cornell *et al.*, 1997). Although CLA's anticarcinogenic effects are well established, potential mechanisms of action in reducing tumorigenesis have yet to be elucidated.

Ip *et al.* (1991) demonstrated that CLA reduces tumor multiplicity in a rat model of mammary carcinogenesis suggesting that CLA may affect tumor metastasis. In support of this, Visonneau *et al.* (1997) used a transplantable metastatic human mammary tumor line (MDA-MB-268) injected into SCID mice fed basal diets or basal diets supplemented with 1% (w/w) CLA. CLA was found to completely eliminate metastasis to secondary sites (i.e. the lungs, bone marrow, and blood) as compared to controls, clearly indicating potent effects in the metastatic process. This group also found that CLA-fed mice had highly undifferentiated tumor cells lacking organization into rosettes or ducts.

An important factor in facilitating metasasis is adhesion, between tumor cells and between tumor cells and the extracellular matrix. The effects of CLA on adhesion have not yet been studied, however research examining the modulatory effect of other fatty acids on adhesion (Lu *et al.*, 1995; Johanning and Lin, 1995; Collie-Duguid and Wahle, 1996) support this hypothesis for the anti-metastatic mechanism of dietary lipids.

Despite the differential effects on adhesion which have been found, it is clear from the literature that EPA and DHA reduce tumor metastasis and that ω-3 fatty acids affect cell adhesion function and expression. Conversely, it has been found that LA also enhances tumor metastasis and tumor cell adhesion. In light of the opposing effects of LA and CLA and the similar effects of EPA/DHA and CLA on tumor growth, development and metastasis, it seems logical that CLA as a dietary fat may affect tumor metastasis by modulating cell adhesion.

The purpose of the following experiment was to examine the incorporation of CLA into tumor cell membranes and its effects on expression and function of adhesion molecules as a potential mechanism of reducing metastatic spread in mammary carcinogenesis.

II. Materials and Methods

A) Cell Line Maintenance

The MDA-MB-231 cell line (human mammary adenocarcinoma) was obtained from the American Type Culture Collection (Rockville, MD) and maintained in Iscove's modified Dulbecco's medium with 1% (v/v) antibiotic/antimycotic and 5% (v/v) FCS (all from Gibco BRL, Grand Island, NY). Media was changed every 3 days until monolayers reached 90% confluency (determined by viewing monlayers under a light microscope) at which time cells were passaged to new flasks. Cells were cultured at 37°C in 5% CO_2 at 98% relative humidity in a sterile incubator (Model 3187, Forma Scientific).

B) Cell Proliferation

To study cell growth, cells were seeded to 24-well, flat-bottom tissue culture treated plates (2×10^4 cells/well; Corning, New York, NY) and cultured for two days to allow formation of monolayers. Media was then replaced with fatty acid-supplemented media. The rationale for the fatty acid concentrations used is outlined by the results of preliminary experiments described in ***Appendix E***. This media was prepared by adding appropriate levels of fatty acid (LA and CLA synthesized as described by Ma *et al.* (in press) dissolved in ethanol into sterile tubes (15 mL; Sarstedt Inc., Newton, NC). Ethanol was then evaporated under $N_{(g)}$. Separately, 0.1% (w/v) bovine serum albumin (fraction V BSA; Sigma Chemical Co., St. Louis, MO) was incubated with media (prepared as described above except 1% rather than 5% (v/v) FCS) for 30 minutes in a shaking water bath at 37°C to dissolve BSA. This media was then sterile syringe-filtered (0.22 µm, Millex®-GS; Millipore Corp., Bedford, MA) and added to the sterile tubes containing fatty acid at appropriate volumes to create the desired concentration. These fatty acid solutions were again incubated for 30 minutes in a shaking water bath at 37°C to solubilize the fatty acids via BSA binding. To replace media with the fatty acid solutions, the original media in each well was gently removed under sterile conditions with a pipette. The freshly prepared fatty acid solutions were then added to each well (1 mL) and allowed to incubate for 2 days.

Cell proliferation was determined using ^{3}H-thymidine incorporation. At 48 hours, cells were pulsed with ^{3}H-thymidine (5 μCi; Amersham, Ontario, Canada) for 4 hours. Media was then gently removed from each well and 300 μL of trypsin-EDTA (Gibco BRL, Grand Island, NY) was added. Cells were incubated with trypsin for 5 minutes at 37°C to disrupt cell adhesion to the plate. Phosphate buffered solution (PBS; 600 μL) was then added and mixed thoroughly in each well to evenly suspend cells. Aliquots (200 μL in quadruplicate) were transferred to non-sterile non-tissue culture treated 96 well V-bottom plates (Costar, Cambridge, MA). Cells were immediately harvested onto glass fiber filter mats (Skatron, Suffolk, UK) with a multiwell harvester (Skatron, Lier, Norway), transferred to scintillation vials containing Ecolite® and counted on a beta counter (Beckman 5000, Beckman Instruments, Palo Alto, CA). The total dpm for each 200 μL aliquot were used to determine thymidine incorporation and estimate the proliferative response.

C) Cell Adhesion Assay

Cells were seeded into sterile tissue culture treated petri dishes (1×10^6 cells, 140 mm diameter, Nunclon®, Nalge Nunc International, Denmark) and allowed to adhere for two days. Media was then replaced by fatty acid-supplemented media (prepared as described above) and cells were allowed to incubate for two days. After one day of incubating cells with the fatty acids, preparation of the plates for the adhesion assay (as described by Johanning and Lin, 1995) was begun. Briefly, collagen IV, fibronectin (both from Sigma Chemical Co., St. Louis, MO), and Matrigel® (Collaborative Biomedical Products, Bedford, MA) were diluted in sterile PBS to a concentration of 200 μg/mL and 50 μL/well was added to tissue culture treated, flat bottom 96 well plates (Nuncatom, Nalge Nunc International, Denmark). Plates were incubated for 2 hours at 37°C and subsequently washed twice with 200 μL of PBS. A BSA solution was then added (100 μL of 5% BSA (w/v)) and allowed to incubate with the substrates for 30 minutes at room temperature to allow non-specific binding to occur. Plates were again washed with 200 μL of PBS and stored overnight at 4°C.

After cells had incubated for 48 hours with the fatty acid solutions they were removed from petri dishes by gently scraping with a rubber policeman and transferring into 50 mL tubes (Sarstedt Inc., Newton, NC). Cells were washed by centrifuging at 1000 rpm for 10 minutes, removing the supernatant, and resuspending in PBS. This washing process was repeated, and cells were resuspended in media supplemented with 0.1% (w/v) BSA. Cells were counted and viability was examined by trypan blue exclusion. Cells were diluted in media + 0.1% (w/v) BSA to a concentration of 3.5 x 10^5 cells/mL. A 100 μL aliquot of this solution was added to each well (including blank wells without substrate) and allowed to incubate for 90 minutes at 37°C. The plate were then washed by adding 200 μL of PBS, inverting and removing excess on an absorbent surface. This washing procedure was repeated three times. Crystal violet dye (50 μL, 0.5% (w/v) solution) was added to each well and allowed to sit for 10 minutes at room temperature. Subsequently, excess dye was removed by immersing the plate three times in tap water. The plate was then dried overnight.

The following day, 100 μL of 0.1M sodium citrate in 50% ethanol was added to each well. After 20 minutes, the optical density was read on a multiwell plate reader (540 nm- absorbance, 405 nm- reference; Model EL309; Bio-tek Instruments Inc., Burlington, VT). Optical density was used as a measure of adhesion of cells to the substrates or to the plastic plate alone (used as the blank). All measurements were made in triplicate.

D) Tumor Cell Phenotyping

Tumor cell expression of integrins were determined by immunofluorescence using mouse monoclonal antibodies directed against the extracellular portion of human β_1 (CD29) and β_4 (CD104) integrins. The immunofluorescent label (fluoroscein isothiocyanate, FITC) was conjugated to goat anti-mouse $IgG_{1,\lambda}$ antibodies. The phenotypic assays utilized single labelling only. V-bottom 96 well, non-sterile, non-tissue culture treated plates (Costar, Cambridge, MA) were preconditioned by adding PBS + 4% (v/v) FCS to each well and allowing incubation for at least 15 minutes at room temperature. Cells were gently removed from petri dishes by scraping with a rubber policeman and counted for viability as described above. Aliquots of 0.25 x 10^6 viable cells in PBS + 4% (v/v) FCS were added to wells. PBS + 4% FCS was then added to fill each well and cells were pelleted by centrifugation at 1250 rpm (480 g) for 3 minutes at 4°C. Plates were inverted to remove fluid, followed by vortexing to break up the pellet. Antibodies (2 μg/10^6 cells for CD29, 0.5 μg/10^6 cells for CD104, both from Pharmingen, San Diego, CA) or PBS + 4% FCS (to control wells) were subsequently added and allowed to incubate at 4° for at least 20 minutes. Cells were washed by adding PBS + 4% FCS, centrifuging for 3 minutes at 4°C, removing fluid by inverting the plate and vortexing to break up the pellet. This washing was repeated three times. FITC (1:300 dilution; Cedarlane® Laboratories Limited, Hornby, ON) was then added to each well and allowed to incubate at 4°C for at least 20 minutes. Cells were washed three times as described above. Cells were fixed in 200 μL of PBS + 1% paraformaldehyde (Anachemia Science, Montreal PQ), acquired by flow cytometry on a FACScan (Becton Dickinson, Sunnyvale, CA) and analyzed (Becton Dickinson, Sunnyvale, CA) using the CellQuest software program.

To analyze, viable cells were gated by plotting cells based on size (forward scatter; FSC-H) and granularity (side scatter; SSC-H, ***Figure 4.1***).

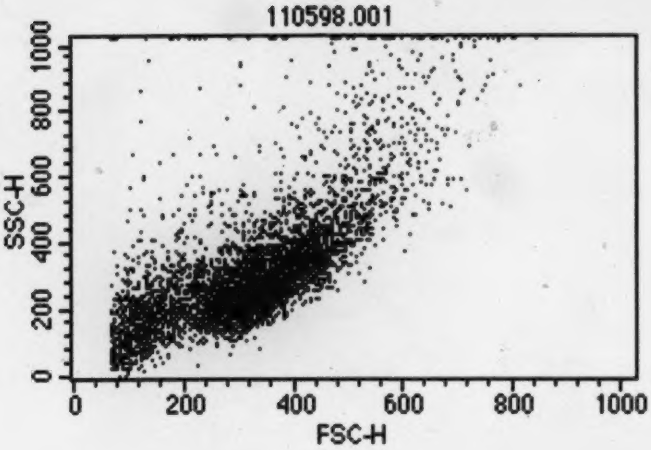

Figure 4.1. Forward Scatter (Size) versus Side Scatter (Granularity) of MDA-MB 231 Cells to Allow Elucidation and Gating of the Viable Cell Population.

Background fluorescence was determined by incubating cells with FITC-conjugated goat anti-mouse $IgG_{1,\lambda}$ only.

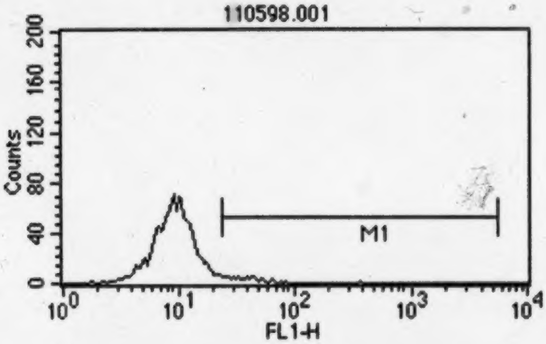

Figure 4.2. Binding of Fluoroscein isothiocyanate-Conjugated Goat Anti-mouse $IgG_{1,\lambda}$ to MDA-MB 231 Cells to Determine Background Fluorescence. *"M1" represents the region of binding chosen to represent positive binding to cell markers.*

For both the CD29 (***Figure 4.3)*** and CD104 (***Figure 4.4***) markers, there were no background fluorescent peaks. Therefore, it was necessary to ensure that the single peak seen was a true positive peak and not a shift in the background fluorescence peak. This was particularly important for the CD104 peak which displayed considerable overlap with background fluorescent levels.

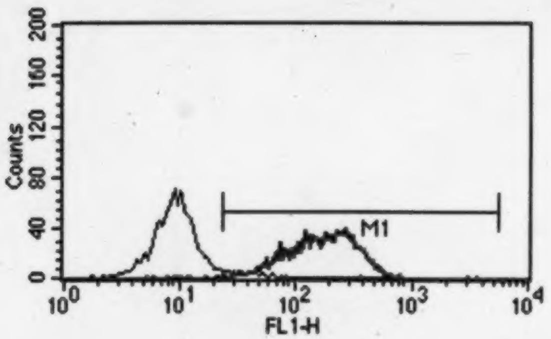

Figure 4.3. CD29+ Binding versus Binding of Fluoroscein Isothiocyanate-Conjugated Goat Anti-mouse IgG$_{1,\lambda}$ to MDA-MB 231 Cells. *The thinner line represents background fluorescence of FITC binding to MDA-MB 231 cells whereas the thicker line represents the positive binding to the CD29 marker. Virtually no overlap was noted.*

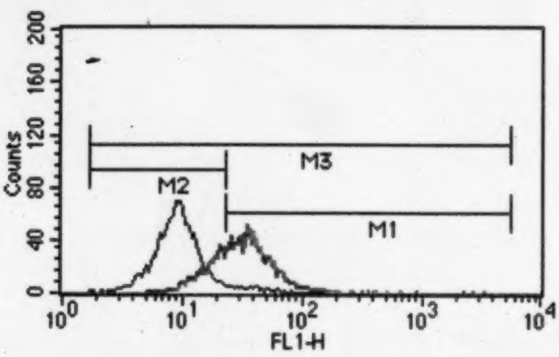

Figure 4.4. CD104+ Binding versus Binding of Fluoroscein Isothiocyanate-Conjugated Goat Anti-mouse IgG$_{1,\lambda}$ to MDA-MB 231 Cells. *The thinner line represents background fluorescence of FITC binding to MDA-MB 231 cells whereas the thicker line represents the binding to the CD104 marker. Some overlap of the peaks was observed.*

According to the Kolmogorov-Smirnov statistic which tests for the presence of two distinct populations, both the CD29 ($p \leq 0.001$) and CD104 ($p \leq 0.001$) populations were true positive peaks, and not mere shifts in background fluorescence. The single peak indicated that 100% of MDA-MB 231 cells expressed these 2 markers which is consistent with previous studies (Gui *et al.*, 1995; Jones *et al.*, 1997). Therefore mean fluorescence was examined to estimate the density of marker expression on the cell surface. This was accomplished by obtaining the mean fluorescence of a marker which spanned the enter positive peak (M3; see ***Figure 4.5***).

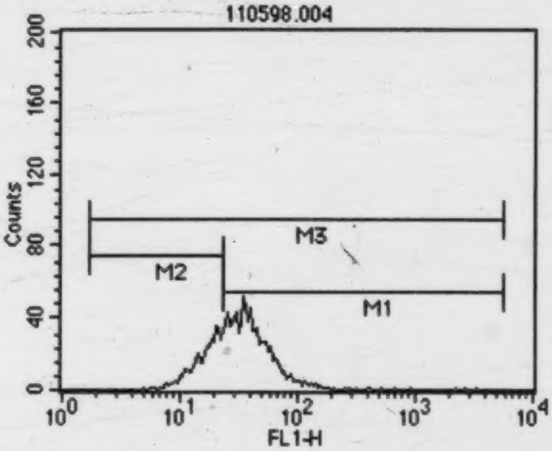

Figure 4.5. Regions Used in Analysis of CD104+ Binding to MDA-MB 231 Cells. *"M1" represents the region of binding initially chosen to represent positive binding to cell markers based on non-specific binding of FITC to MDA-MB 231 cells. Due to statistical calculations indicating that the peak was positive for CD104, this region was not needed for analysis but might be viewed as an arbitrary designation for cells which demonstrated a higher level of CD104 expression on their cell surface. "M3" represents the region chosen to represent the entire positive peak. This region was used for mean fluorescence calculations. "M2" represents the difference between M3 and M1 and might also be seen as the arbitrary designation for cells that demonstrated a lower level of CD104 expression on their cell surface.*

E) Tumor Phospholipid Analysis

A modified Folch method (Folch *et al.*, 1957) was performed to extract lipid from tumor cells which had been incubated with fatty acids. Briefly, 1.6 mL of 0.1 M KCl was added to tubes containing tumor cell pellets. This was vortexed and the suspended cells were transferred to a clean 15 mL methylation tube. KCl (1.6 mL), methanol (0.8 mL), chloroform: methanol (1:1; 2.0 mL), chloroform (2.7 mL), and 2:1 chloroform: methanol (2.5 mL) were added sequentially with vortexing after each addition. Samples were then capped and vortexed for 1 minute and left overnight at 4°C to separate. The next day, the bottom layer was removed, transferred to a clean methylation vial, and dried under $N_2(g)$. An additional 1 mL of chloroform was added to the original tube, vortexed and the bottom layer again transferred once the phases had separated. Chloroform (100 μL) was added to the dried sample which were then run on silica gel H plates (Fisher Scientific Inc, Nepean, ON).

H plates were heat activated at 110°C for 1 hour prior to spotting phospholipid samples. Samples were run in a 30: 9: 25: 6: 18 (by volume) chloroform: methanol: 2-propanol: 0.25% KCl: triethylamine solvent system in a 13 x 13 cm chamber at room temperature for approximately 45 minutes. Plates were dried, sprayed with 0.1% (w/v) ANSA, and bands were identified under ultraviolet light. Bands were then scraped and added to methylation vials. Phosphatidylinositol (PI), sphingomyelin (SM) and phosphatidylserine (PS) bands were stored at -70°C for later methylation and analysis. Phosphatidylcholine and phosphatidylethanolamine (PE) were methylated immediately using a modified methylation procedure (Shantha *et al.*, 1993; Christie, 1982). Briefly, the lipid was dissolved in 0.5mL of tetrahydrofuran (THF). Sodium methoxide (1mL, 0.5M) was added, samples were vortexed and heated to 50°C for 10 minutes. Glacial acetic acid (50 µL) was added, samples were vortexed, 5 mL of ddH_2O were added and samples were again vortexed. Lipid was extracted twice with 2.5mL of hexane, transferred to clean, dry GC vials and dried under $N_2(g)$. Dried samples were flushed with nitrogen and stored at -70°C until analysis. Analyses were carried out on a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d. x 0.2 µm film thickness; Supelco Inc, Bellefonte, PA) by dissolving samples in hexane and setting the column conditions as follows:

injector port: 250°C	detector temperature: 270°C
gas carrier: helium	column pressure: 50 psi
40: split mode	

Samples were eluted over 110 minutes using temperatures from 130°C to 225°C. By this method, all saturated, monounsaturated and polyunsaturated FAME ranging from C14 to C24 were separated. Several isomers of CLA were also separated by this column (see ***Figure 3.2***).

F) Statistical Analysis

All statistics were performed using the SAS analysis program (SAS 6.11, SAS

Institute Inc., Cary, NC). Differences in cell proliferation due to fatty acid incubation were analyzed by one-way ANOVA. Differences in cell adhesion were analyzed by one-way ANOVA with a block for plate to account for plate to plate variation. Cell phenotyping was analyzed by one-way ANOVA with a block for day. Unless otherwise specified, statistical differences were determined by examining the least square means with statistical significance set at a value of $p \leq 0.05$. Values are expressed as mean ± SEM.

III. Results

A) Cell Proliferation

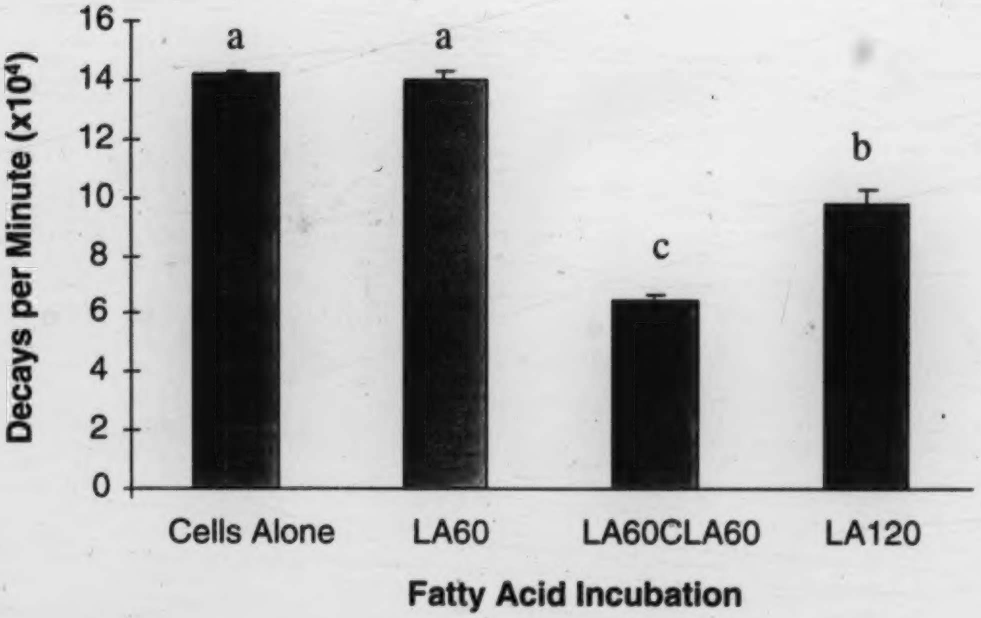

Figure 4.6. Proliferation of MDA-MB 231 Cells After a 48 Hour Incubation With Various Fatty Acids. *Bars represent mean ± SEM with n=6 for each treatment. Different letters on eact bar indicate significant differences between treatments ($p<0.05$).*

For cells incubated with fatty acids, LA at a 60 μM concentration resulted in the greatest proliferation (***Figure 4.6***). When the LA concentration was doubled to 120 μM, proliferation significantly decreased. However, the treatment providing 60 μM each of LA and CLA resulted in the lowest ($p<0.05$) rate of proliferation of tumor cells.

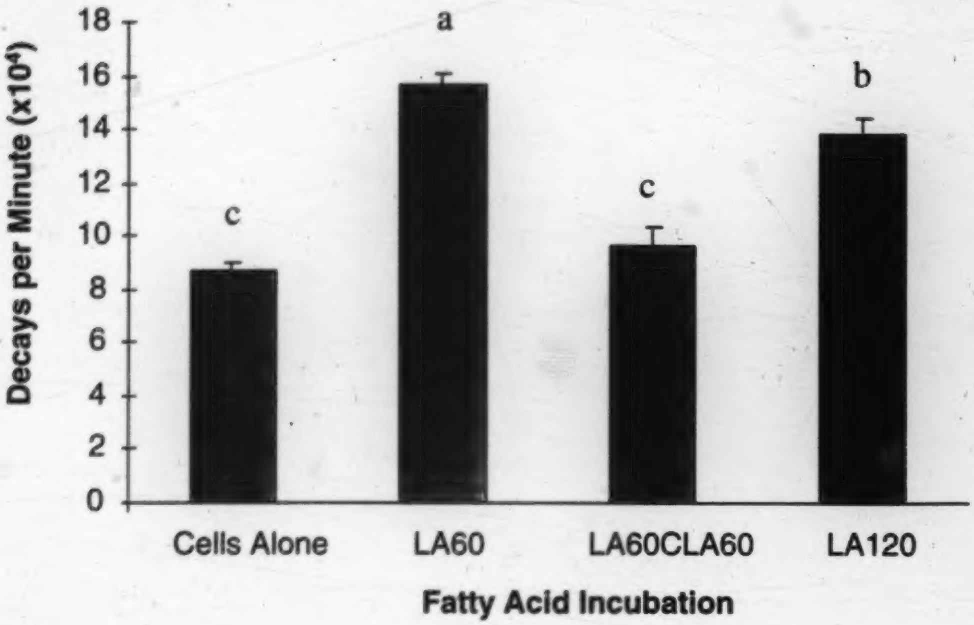

Figure 4.7. Proliferation of MDA-MB 231 Cells After a 72 Hour Incubation With Various Fatty Acids. *Bars represent mean ± SEM with n=6 for each treatment. Different letters indicate significant differences between treatments ($p<0.05$).*

When this proliferation assay was repeated with a 72 hour fatty acid incubation, the same relative trends as ***Figure 4.6*** were observed for the cells exposed to LA or LA + CLA (***Figure 4.7***). There was a reduction in the proliferation of cells alone (without fatty acids) compared to proliferation at 48 hours. This may have been due to a reduction in essential nutrients (eg. LA) in the media after a longer period of incubation. However, this reduction in proliferation did not change the important trends as "cells alone" did not serve as our control, but rather "LA60".

B) Cell Adhesion

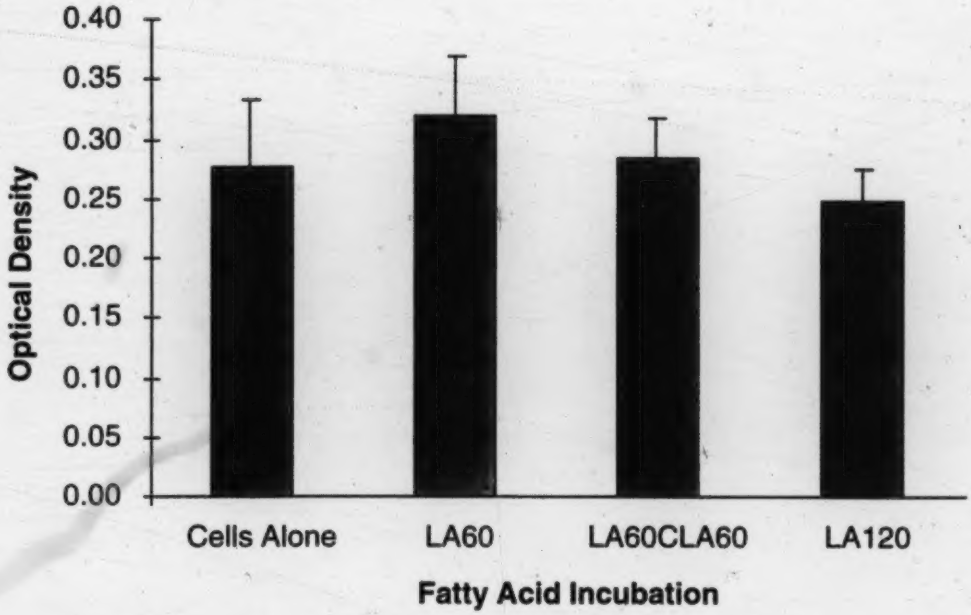

Figure 4.8. Adhesion of MDA-MB 231 Cells to Collagen IV After a 48 Hour Incubation with Various Fatty Acids. *Bars represent mean ± SEM with n=6 for each treatment.*

No significant differences in adhesion to collagen IV were noted after incubating cells for 48 hours with various fatty acids (***Figure 4.8***).

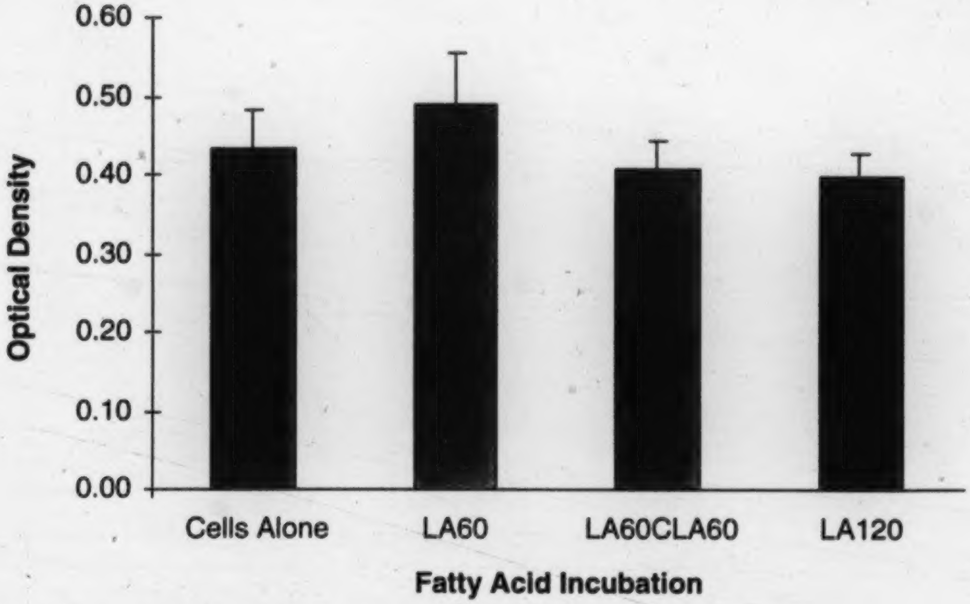

Figure 4.9. Adhesion of MDA-MB 231 Cells to Fibronectin After a 48 Hour Incubation with Various Fatty Acids. *Bars represent mean ± SEM with n=6 for each treatment.*

No significant differences in adhesion to fibronectin were noted after incubating cells for 48 hours with various fatty acids (***Figure 4.9***).

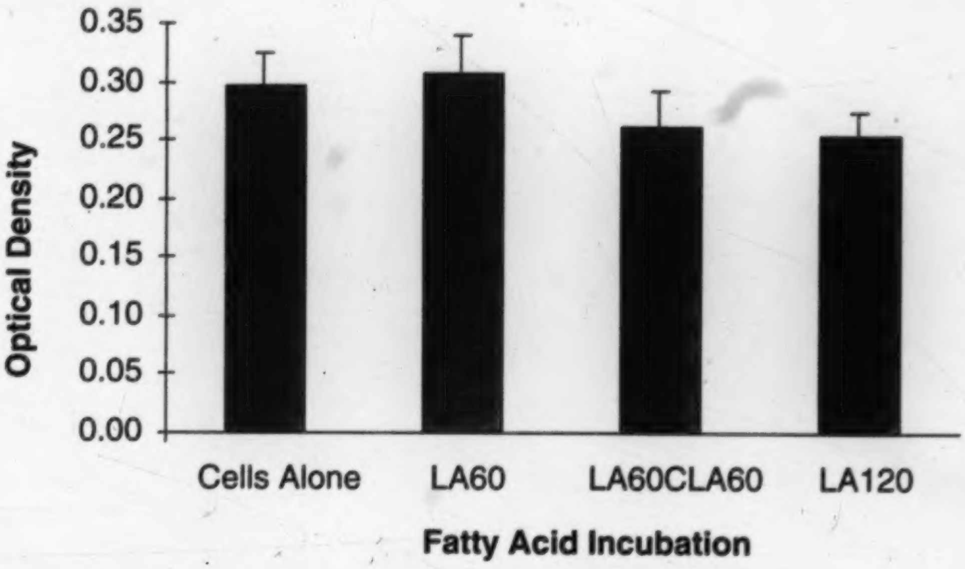

Figure 4.10. Adhesion of MDA-MB 231 Cells to Matrigel After a 48 Hour Incubation with Various Fatty Acids. *Bars represent mean ± SEM with n=6 for each treatment.*

No significant differences in adhesion to Matrigel were noted after incubating cells for 48 hours with various fatty acids (***Figure 4.10***).

C) Cell Phenotyping

Fatty Acid Incubation (μM)	***Mean Fluorescence***		
	FITC background	***CD29+***	***CD104+***
Cells Alone	11.0 ± 0.4	172 ± 18^{b}	31.0 ± 1.5^{b}
LA 60	11.9 ± 0.4	155 ± 12^{ab}	25.8 ± 1.5^{a}
LA 60 CLA 60	12.1 ± 0.4	127 ± 15^{a}	24.6 ± 1.4^{a}
LA 120	12.7 ± 0.6	143 ± 16^{ab}	24.1 ± 1.0^{a}

Table 4.1. Mean Fluorescence of MDA-MB 231 Cells Expressing CD29 or CD104 Following 48 Hour Incubation with Various Fatty Acids. *Values represent mean ± SEM with n=6 for each treatment. Different superscripts indicate significant differences between treatments ($p<0.05$).*

No differences were noted in mean background fluorescence of MDA-MB 231 cells under the different fatty acid treatments (***Table 4.1***). In addition, there were no differences in mean fluorescence between the three groups which were incubated with fatty acids for either CD29 (β_1 integrin) or CD104 (β_4 integrin). The mean fluorescence of CD29+ cells was, significantly reduced ($p<0.05$) when cells were incubated with 60 μM of LA and CLA compared to cells cultured in media alone (***Table 4.1***). The mean fluorescence of CD104+ cells was lower ($p<0.05$) for all tumor cells incubated with fatty acids compared to those cultured in media alone (***Table 4.1***).

D) Tumor Cell Phospholipid Analysis

Fatty Acid	Fatty Acid Treatment			
	Cells Alone	*LA 60 μM*	*LA CLA 60 μM*	*LA 120 μM*
Saturates				
16:0	16.1 ± 1.1	16.3 ± 2.2	15.8 ± 3.3	17.1 ± 0.7
17:0	0.37 ± 0.15	0.31 ± 0.12	0.45 ± 0.17	0.43 ± 0.12
18:0	18.8 ± 1.2^{c}	13.1 ± 1.6^{b}	6.29 ± 2.32^{a}	11.9 ± 1.3^{b}
Total Saturates	**35.5 ± 1.2^{b}**	**29.1 ± 2.2^{ab}**	**22.6 ± 4.9^{a}**	**29.6 ± 1.3^{ab}**
Monounsaturates				
16:1	3.61 ± 0.33	4.12 ± 1.62	1.10 ± 0.37	2.05 ± 0.50
18:1	33.7 ± 1.8^{b}	15.3 ± 3.2^{a}	13.9 ± 2.6^{a}	17.5 ± 2.5^{a}
20:1	0.13 ± 0.04	0.07 ± 0.04	0.18 ± 0.10	0.37 ± 0.16
Total Monounsaturates	**39.0 ± 0.9^{b}**	**22.1 ± 3.8^{a}**	**15.2 ± 2.4^{a}**	**19.4 ± 2.4^{a}**
Polyunsaturates				
18:2(6)	9.28 ± 2.25^{a}	23.6 ± 5.0^{b}	30.3 ± 7.0^{bc}	41.0 ± 2.3^{c}
18:3(3)	1.66 ± 0.23^{b}	0.73 ± 0.25^{a}	0.82 ± 0.04^{a}	0.22 ± 0.13^{a}
20:2(6)	0.11 ± 0.02^{bc}	0.16 ± 0.04^{c}	0.05 ± 0.03^{ab}	0.01 ± 0.01^{a}
20:3(6)	0.94 ± 0.24^{b}	0.03 ± 0.01^{a}	0.07 ± 0.03^{a}	0.11 ± 0.02^{a}
20:4(6)	7.43 ± 0.38^{c}	3.95 ± 0.62^{b}	1.41 ± 0.17^{a}	3.05 ± 0.40^{b}
20:5(3)	0.48 ± 0.15^{ab}	0.87 ± 0.26^{b}	0.55 ± 0.14^{ab}	0.13 ± 0.06^{a}
22:4(6)	0.15 ± 0.07	0.07 ± 0.05	0.07 ± 0.03	0.13 ± 0.03
22:5(6)	1.25 ± 0.32	0.86 ± 0.23	0.49 ± 0.29	0.49 ± 0.18
22:5(3)	0.83 ± 0.27^{ab}	1.22 ± 0.32^{ab}	0.46 ± 0.24^{a}	1.55 ± 0.40^{b}
22:6(3)	3.66 ± 0.80^{c}	2.26 ± 0.50^{bc}	0.56 ± 0.07^{a}	0.88 ± 0.11^{ab}
Total CLA	2.47 ± 0.62^{a}	1.75 ± 0.47^{a}	32.9 ± 0.8^{b}	1.91 ± 0.40^{a}
Total ω-6	15.6 ± 1.1^{a}	28.3 ± 4.9^{ab}	32.5 ± 7.18^{ab}	37.5 ± 7.6^{b}
Total ω-3	5.47 ± 1.12^{c}	4.50 ± 0.87^{bc}	2.20 ± 0.27^{ab}	2.12 ± 0.38^{a}
Total Polyunsaturates	**24.3 ± 1.1^{a}**	**39.5 ± 5.2^{b}**	**60.1 ± 4.8^{c}**	**48.9 ± 2.2^{b}**

Table 4.2. Fatty Acid Analysis of the Phosphatidylcholine Fraction of MDA-MB 231 Cells Following 48 Hour Incubation with Various Fatty Acids. *Values represent mean ± SEM with $n \geq 3$ for each treatment. Different superscripts indicate significant differences between treatments ($p \leq 0.05$).*

i) LA 60 versus LA+CLA - Adding CLA to the LA resulted in significant incorporation of CLA into the PC fraction (33% w/w) compared to cells incubated in LA alone (2% w/w). CLA incubation also resulted in a decrease in AA (20:4(6)), however there was no effect on total ω-6 fatty acids. In addition, CLA reduced DHA (22:6(3)). Although there was a trend ($p=0.08$), this decrease did not result in a significant reduction in total ω-3 fatty acids. Also, CLA incubation did not reduce levels of LA (18:2(6)) in this fraction.

ii) LA 120 versus LA+CLA- Cells exposed to CLA demonstrated significantly increased incorporation of CLA (33% w/w) compared to cells exposed to an equal fatty acid concentration without CLA (2% w/w). CLA also resulted in decreased levels of AA and 22:5(3), however no effects on LA, total ω-6 or total ω-3 fatty acids were seen. Interestingly, despite exposure to the same total PUFA concentration, cells exposed to CLA exhibited significantly higher total PUFA in the PC fraction.

iii) LA 60 versus LA 120- Cells exposed to the higher concentration of LA had an approximate two-fold increase in LA content, a decrease in 20:2(6) and EPA (20:5(3)) as well as a decrease in total ω-3 fatty acids.

Fatty Acid	Fatty Acid Treatment			
	Cells Alone	*LA 60 μL*	*LA CLA 60 μL*	*LA 120 μL*
Saturates				
16:0	1.03 ± 0.40	1.34 ± 0.50	4.53 ± 2.01	5.75 ± 2.75
17:0	0.26 ± 0.14	0.10 ± 0.09	0.38 ± 0.38	0.14 ± 0.06
18:0	8.90 ± 1.22^{ab}	6.32 ± 1.34^{a}	8.06 ± 1.74^{ab}	11.4 ± 0.6^{b}
Total Saturates	**9.62 ± 1.74^{ab}**	**7.71 ± 1.20^{a}**	**14.5 ± 4.5^{ab}**	**16.9 ± 3.0^{b}**
Monounsaturates				
16:1	0.91 ± 0.12^{ab}	2.09 ± 0.77^{b}	1.07 ± 0.21^{ab}	0.75 ± 0.21^{a}
18:1	23.0 ± 4.3^{ab}	27.9 ± 2.9^{b}	16.8 ± 3.0^{a}	20.4 ± 2.5^{ab}
20:1	3.41 ± 1.38	2.74 ± 1.60	0.73 ± 0.37	1.52 ± 0.56
Total Monounsaturates	**27.9 ± 2.9^{ab}**	**32.6 ± 3.4^{b}**	**19.5 ± 3.2^{a}**	**23.3 ± 3.1^{a}**
Polyunsaturates				
18:2(6)	4.45 ± 0.96^{a}	13.3 ± 0.7^{b}	15.0 ± 2.2^{b}	26.2 ± 6.0^{c}
18:3(3)	2.12 ± 0.72	3.13 ± 0.41	2.65 ± 0.18	2.70 ± 0.42
20:2(6)	0.05 ± 0.02	0.14 ± 0.08	0.34 ± 0.20	6.23 ± 3.21
20:3(6)	0.26 ± 0.14	0.17 ± 0.09	0.06 ± 0.03	0.13 ± 0.02
20:4(6)	18.7 ± 2.1	13.8 ± 3.4	15.2 ± 3.5	14.8 ± 4.6
20:5(3)	0.13 ± 0.05	0.12 ± 0.04	0.16 ± 0.07	0.30 ± 0.14
22:4(6)	0.10 ± 0.03^{a}	0.28 ± 0.13^{ab}	0.13 ± 0.03^{ab}	0.37 ± 0.11^{b}
22:5(6)	0.08 ± 0.02	0.41 ± 0.20	0.10 ± 0.06	0.46 ± 0.19
22:5(3)	6.18 ± 1.39	7.33 ± 2.30	7.75 ± 2.62	6.61 ± 2.09
22:6(3)	6.60 ± 1.93	6.76 ± 2.63	6.13 ± 2.35	4.49 ± 0.91
Total CLA	1.33 ± 0.24^{a}	1.51 ± 0.21^{a}	16.3 ± 3.2^{b}	1.83 ± 0.39^{a}
Total ω-6	24.0 ± 2.7^{a}	30.9 ± 3.2^{a}	31.0 ± 5.0^{a}	45.1 ± 1.8^{b}
Total ω-3	17.1 ± 1.9	16.7 ± 4.3	16.0 ± 2.8	11.0 ± 2.6
Total Polyunsaturates	**48.5 ± 4.0**	**50.0 ± 6.6**	**61.0 ± 8.2**	**61.2 ± 3.0**

Table 4.3. Fatty Acid Analysis of the Phosphatidylethanolamine Fraction of MDA-MB 231 Cells Following 48 Hour Incubation with Various Fatty Acids. *Values represent mean ± SEM with $n \geq 3$ for each treatment. Different superscripts indicate significant differences between treatments ($p \leq 0.05$).*

i) LA 60 versus LA+CLA - CLA incubation resulted in a significant increase in CLA (16% w/w) compared to LA 60 alone (1.5% w/w). The level of CLA incorporation was half of that seen in the PC fraction however (33% w/w, ***Table 4.2***). A reduction in oleic acid (18:1) was also noted with CLA supplementation. No changes in any ω-6 or ω-3 fatty acids were seen.

ii) LA 120 versus LA+CLA- Again, CLA was significantly increased in the PE fraction of cells incubated with CLA (16% w/w) compared to those incubated with 120 μM LA (1.8% w/w). LA 120 cells exhibited significantly higher levels of LA and increased total ω-6 fatty acids compared to cells exposed to LA+CLA. No differences in AA or total ω-3 fatty acids were noted.

iii) LA 60 versus LA 120- As seen in the PC fraction (***Table 4.2***), the higher concentration of LA resulted in a two-fold increase in LA incorporation in the membrane. The LA120 group also exhibited an increase in total ω-6 compared to the LA 60 group. An increase in LA was found to be associated with an increase in stearic acid (18:0) and total saturated fatty acids. However, the LA 120 group had less 16:1 and less total MUFA. Again, no differences in levels of AA or total ω-3 fatty acids were seen.

Lipid Fraction	*c*9*t*11	*t*10*c*12	*t*9*t*11	others	*c*9*t*11:*t*10*c*12
PC	39.4 ± 1.0	42.1± 6.9	2.64 ± 1.46	16.6 ± 7.7	1.00 ± 0.21
PE	38.2 ± 0.8	54.1 ± 1.0	5.65 ± 1.12	2.11 ± 0.43	0.71 ± 0.03

Table 4.4. Conjugated Linoleic Acid Isomer Analysis of the Phosphatidylcholine and Phosphatidylethanolamine Fractions of MDA-MB 231 Cells Following 48 Hour Incubation with 60 μM each of Linoleic and Conjugated Linoleic Acid. *Values represent mean percent of total CLA ± SEM with n=3 for each lipid fraction.*

Isomer distribution in the PC fraction was similar to the isomer distribution of the stock CLA used to create the fatty acid mixture (see ***Figure 3.2***). The ratio between the two major isomers (*c*9*t*11:*t*10*c*12) was the same as that seen in the stock CLA. That is, a ratio of approximately 1.0 was obtained for PC. In the PE fraction, there was a relative increase in *t*10*c*12 and a relative decrease in *c*9*t*11 as evidenced by the reduced ratio between the two isomers (0.71 ± 0.03).

IV. DISCUSSION

A) Tumor Cell Proliferation

Previous studies have examined the effects of various fatty acids on human mammary tumor cell growth and metastases. Rose *et al.* (1994) demonstrated that the injection of MDA-MB 231 cells into nude mice resulted in larger tumors if the mice were fed 12% (w/w) LA versus 2% LA with the total fat content held constant at 23% (w/w). The 12% LA group also had a higher incidence of microscopic metastases. Similar findings have been noted by other researchers (Hubbard and Erickson, 1987; Karmali *et al.*, 1993). Conversely, increasing dietary levels of ω-3 fatty acids, particularly those found in fish oil, has been shown to reduce tumor cell growth and metastasis in animal tumor models (Karmali *et al.*, 1993; Rose *et al.*, 1995).

Similar trends have been found when examining the effects of fatty acids on tumor cell growth and proliferation *in vitro*. For the purposes of the present study, the MDA-MB 231 cell line is of particular interest. The MDA-MB 231 is a human mammary adenocarcinoma cell line that is vimentin-negative, estrogen-receptor negative, and highly aggressive (Thompson *et al.*, 1992). The growth of these cells has previously been shown to be maximally stimulated by LA at a concentration of 0.75 μg/mL or approximately 3 μM, and inhibited by DHA and EPA at similar concentrations (Rose and Connolly, 1990). Another study noted inhibitory effects of 0.1-8.1 μg/mL DHA (0.3-24.7 μM) or EPA (0.3-26.8 μM) on cell growth of MDA-MB 231 in the presence or absence of 625 ng/mL LA (2.2 μM; Noguchi *et al.*, 1995). Therefore, it was known that this cell line was responsive to various fatty acids implicated in tumorigenesis. Nonetheless, it was still necessary to establish appropriate media conditions as cell growth may be affected by numerous factors including the concentration of FCS (Mæhle *et al.*, 1995), ethanol (Angeletti and de Alaniz, 1996) and BSA (desBordes and Lea, 1995). Because ethanol is known to affect the cell membrane (Chen *et al.*, 1996), cell conditions were established in the absence of this compound (see ***Appendix E***).

Inhibitory effects of CLA on human tumor cell lines (Cesano *et al.*, 1998; Yoon *et al.*, 1997; Seaman *et al.*, 1992; Shultz *et al.*, 1992A,1992B), including mammary tumor cell lines (Cunningham *et al.*, 1997; Visonneau *et al.*, 1997) have been previously noted *in vivo* and *in vitro* by several investigators. In addition, 1% (w/w) dietary CLA has been demonstrated to reduce tumor metastasis (Cesano *et al.*, 1998; Visonneau *et al.*, 1997) in sites such as lung, blood and bone marrow. In light of the similar effects of ω-3 fatty acids (DHA and EPA) and CLA on tumor development, it is reasonable to postulate that they may work through a similar anticarcinogenic mechanism.

The growth and development of tumor cells rely on the presence of the essential fatty acid, LA (Ip *et al.*, 1985). For this reason, in our study, LA was present in all treatments to ensure that reductions in proliferation due to CLA were not merely due to an absence of LA. Effects of fatty acids also vary with concentration such that higher concentrations may become cytotoxic to cells (Rose and Connolly, 1990; Tolnai and Morgan, 1962). Therefore, to ensure that the 60 μM LA + 60 μM CLA treatment did not reduce proliferation due to a cytotoxic level of fatty acids, an additional control was used (LA 120 μM) which contained an equal total fatty acid concentration to the CLA treatment. The concentration of CLA chosen (60 μM) was based upon previous studies which demonstrated inhibitory effects at this level (Cunningham *et al.*, 1997; Seaman *et al.*, 1992; Shultz *et al.*, 1992A, 1992B). Levels of LA were based on a preliminary study examining proliferative effects over a range of different concentrations (see ***Appendix E***). The levels of LA that were used in the present study were somewhat higher than previous studies which demonstrated maximal proliferation of MDA-MB 231 cells at approximately 3 μM (Rose and Connolly, 1990). However in these other studies, the culture conditions (media, level of BSA and FCS, presence of ethanol) and the method of delivery of fatty acids were different from our study which may have accounted for the sensitivity to very low concentrations of these lipids.

Our results clearly demonstrate an effect of adding CLA to LA on cell proliferation (***Figures 4.6*** and ***4.7***). LA (60 μM) resulted in the highest estimated rate of proliferation. The proliferation rate decreased significantly when LA concentrations were increased to 120 μM, indicating some inhibition of tumor cell growth at higher concentrations of LA. This might be explained by the toxic effect of fatty acids on cells (Jiang *et al.*, 1998). However, adding CLA to LA resulted in the greatest decrease in tumor cell growth. Growth inhibition was also significantly greater (p=0.0001) than the LA 120 μM suggesting the effect was more than just due to the total fatty acid concentration on cell growth. The same effects on growth were observed after both 48 (***Figure 4.6***) and 72 hour (***Figure 4.7***) incubation. By including the LA control cells, we demonstrated that CLA exerts inhibitory effects on the growth of a human mammary adenocarcinoma cell line and that this inhibition was not merely due to a lack or LA or to cytotoxic concentrations of fatty acids.

This is the first time, to our knowledge, that the effects of CLA on the MDA-MB 231 line has been examined. Although our results concur with previous findings regarding the anti-tumorigenic effects of CLA, none of the earlier *in vitro* studies compared the effects of CLA when co-incubated with LA. Therefore one was unable to exclude the possible inhibitory effects due to changes in fatty acid concentration or the absence of the essential growth factor linoleic acid. Our study design allowed the separation of these two factors and confirmed inhibitory effects of CLA independent of effects of LA or fatty acid concentration.

B) Tumor Cell Phospholipid Analysis

The incorporation of dietary fatty acids into cell membranes (Innis and Clandinin, 1981; Garg *et al.*, 1988; Field *et al.*, 1989) including tumor cell membranes (Robinson and Field, in preparation) is well established. In addition, it is known that the phospholipid composition of cells may be modified *in vitro* when different fatty acids are added to culture media (Spector and Yorek, 1985). However, the effects of *in vitro* incubation of CLA on the composition of specific tumor cell phospholipid fractions have not been previously studied. In this respect, the present study provided novel information, specifically examining the PC and PE fractions of MDA-MB 231 human mammary tumor cells.

Although individual tumor phospholipid fractions had not been examined prior to the present study, Ip *et al.* (1991) examined incorporation of dietary CLA (≤1.5% w/w) into tumor cell total phospholipids. This group found that the *c9t*11 isomer was incorporated in proportion to dietary intake and that the tumor cell phospholipids showed no evidence of having reached maximal CLA incorporation at the CLA levels studied. At the highest level of dietary CLA tested (1.5% w/w), CLA composed 0.13% (w/w) of tumor cell total phospholipids.

i) Phosphatidylcholine Fraction- In the following discussion, it is important to note that the "CLA" treatment group refers to cells which were exposed to 60 μM of both CLA and LA. The rationale for using the combination of these two fatty acids was described above in the discussion on tumor cell proliferation. In the current study, fatty acid analysis of the PC fraction (***Table 4.2***) indicated that cells incubated with CLA demonstrated significantly higher levels of CLA (16-fold increase) than did cells which were not incubated with CLA ($p \leq 0.0001$). In addition, CLA treatment reduced levels of AA (20:4(6)) by greater than 50% compared to cells treated with either LA concentration. CLA also reduced levels of DHA (22:6(3)) by more than four-fold compared to the 60 μM LA group and reduced levels of 22:5(3) (approximately 60% reduction) compared to the 120 μM LA group. Surprisingly, however, no effects on LA, total ω-6 or ω-3 fatty acids were noted suggesting a lack of displacement of these fatty acids or fatty acid classes in the membrane by CLA. Displacement of other fatty acids (i.e. LA, LNA) by CLA has been studied by several researchers in different tissues with conflicting results. Whereas the examination of rat mammary tissue (Ip *et al.*, 1996; Ip *et al.*, 1994), rat colon (Liew *et al.*, 1995) and mouse forestomach (Ha *et al.*, 1990) suggested no displacement of LA in phospholipids, rat fat pad (Cook *et al.*, 1993) and hepatic NL (Belury and Kempa-Steczko, 1997) indicated a reduction in LA with dietary CLA supplementation. Our results support a lack of displacement of LA in the membrane PL with CLA incubation, however competition in metabolic pathways can not be excluded. The consistently lower levels of AA and the reductions in certain ω-3 fatty acids suggest that CLA may interfere or compete with these fatty acids. AA and EPA are substrates in the cyclooxygenase and lipoxygenase pathways to produce prostaglandins, leukotrienes, thromboxanes and other metabolites involved in various processes including modulating immune response (Calder, 1998; Kinsella *et al,* 1990). This may indeed be a mechanism of CLA in reducing tumor growth. In addition, studies by Atsumi *et al.* (1997) and Iida *et al.* (1998) provide a potential reason for reductions in AA. These researchers found that the release of AA from tumor cells was enhanced in association with apoptosis, perhaps due to enhanced activity of PLA_2. Another study by Herrmann and colleagues (1997) demonstrated that membrane AA significantly suppressed apoptosis induction in prostate carcinoma cells. These studies may suggest a role for lower levels of AA in the reduced growth of MDA-MB 231 cells by CLA. However, it can not be determined whether reductions in AA would be a cause or effect of apoptotic changes in tumor cells.

In the present study, it was noted that both AA and DHA concentrations in PC were altered with CLA supplementation. Because the intermediates in the pathway converting LA to AA and EPA to DHA do not exhibit a clear pattern (i.e. increased or decreased levels), it is impossible to speculate as to where CLA might interfere, if at all. Further pulse-chase studies using radiolabelled fatty acids would assist in clarifying these ambiguities.

Differences in PC composition were also notable between the two groups exposed to high and low LA concentrations. A two-fold increase in media LA resulted in an approximate two-fold increase in PC LA (***Table 4.2***). Surprisingly this did not result in increased tumor growth. Increasing LA also decreased levels of EPA as well as total ω-3 fatty acids. It has been well established that competition between ω-6 and ω-3 fatty acids exists and our results are consistent with this. Previous literature may also explain the similar levels of AA between the two groups. One of the desaturase enzymes, Δ6 desaturase, which converts LA (18:2(6)) to 18:3(6) is thought to be rate limiting (reviewed by Grammatikos *et al.*, 1994). Therefore, if the V_{max} of Δ6 desaturase in the PC fraction is achieved when substrate (LA) is provided at 60 μM, no further increases in subsequent metabolites (e.g. AA) would be seen when higher levels of LA were used.

ii) Phosphatidylethanolamine Fraction - CLA treatment of cells resulted in significantly higher (16% w/w) incorporation of CLA in the PE fraction than cells not incubated with CLA (<2% w/w). Incorporation of CLA in this fraction was only half of that in the PC fraction (33%, ***Table 4.2***). In agreement with our findings in the PC fraction (***Table 4.2***), cells exposed to CLA had similar levels of LA to cells which were incubated with 60 μM LA only. This again supports the hypothesis that CLA does not displace LA from the membrane. As well, no changes in AA or ω-3 fatty acids were noted which contrasts our findings in the PC fraction (***Table 4.2***) and suggests that CLA does not interfere in desaturation/elongation pathways.. Such contrasting findings seem plausible as the different phospholipid fractions have different compositions and are metabolically unique. For example, the phosphatidylinositol (PI) fraction is unique in its role in second messenger signaling through cleavage of inositol triphosphate and diacylglycerol.

Differences in PE composition were also found between the two groups exposed to high and low LA concentrations. A two-fold increase in media LA concentration resulted in a two-fold increase in PE LA concentration (***Table 4.3***). This was accompanied by an increase in total ω-6. Increasing LA led to higher levels of LA and total ω-6 in the PE fraction whereas LA, but not total ω-6, increased in the PC fraction (***Table 4.2***). This suggests that 60 μM LA may not be sufficient for maximal incorporation of total ω-6 in the PE fraction but that it may be within the PC fraction. Such results again indicate the metabolic uniqueness of different fractions and the importance of examining each fraction individually when studying membrane composition. Finally, the high LA group had an approximate 40% reduction in total MUFA compared to the low LA group. However, the CLA group had similar significantly lower total MUFA than did the low LA group. Such decreases in total MUFA with CLA or high LA treatment might therefore be due to an increase in PUFA concentration, rather than the effect of a specific fatty acid. In light of the structural similarities between certain fatty acids (e.g. *c*9*t*11 isomer and oleic acid), it seems reasonable that replacement of one (oleic) for another of greater abundance in the media (*c*9*t*11 isomer) might occur.

iii) CLA Isomer Distribution in Phospholipid Fractions- A more detailed examination of the major CLA isomers in the PC fraction of cells incubated with CLA reveals a similar overall profile as the stock CLA used to create the fatty acid media (***Table 3.2***). Specifically, the ratio between the two major isomers (*c9t*11 and *t*10*c*12) did not change from that which was administered in the media. This is in contrast to previous dietary studies (Ha *et al.*, 1990; Benjamin *et al.*, 1990) on lipids including mammary tumor PL (Ip *et al.*, 1991) and adipose tissue neutral lipids (Chapter 3) which found a preferential incorporation of the *c9t*11 isomer and, therefore, postulated this to be the biologically active form. Not only did the present study fail to show preferential incorporation, but the *c9t*11: *t*10*c*12 ratio in the PE fraction indicated a preferential incorporation of the *t*10*c*12 isoform. However, it is impossible to determine whether the lower ratio is due to enhanced incorporation of the *t*10*c*12 isomer or enhanced metabolism of the *c9t*11 isomer. In direct contrast, our diet study indicated a preferential incorporation of the *c9t*11 isomer in adipose TG (***Figure 3.18***). Collectively, such results indicate different incorporation of CLA isomers in different tissues and lipid fractions. Future studies using radiolabelled individual isomers may further clarify the incorporation and metabolic fates of CLA isomers in various tissues and lipid classes, as well as their effects on other fatty acids.

iv) Lipid Summary- By examining the fatty acid analysis data for the PC and PE fractions of MDA-MB 231 tumor cells, one can readily determine that the fatty acids in the incubation media were incorporated by both fractions (***Table 4.2 and 4.3***). With the fatty acid of greatest interest, CLA, incorporation into tumor cell phospholipids (32.9 ± 0.8% in PC and 16.3 ± 3.2% in PE) was considerably higher than that achieved by the dietary study conducted by Ip *et al.* (1991; 0.13% total PL). However, the relevance of comparing CLA levels achieved *in vitro* with those achieved *in vivo* is questionable as *in vitro* models are developed to examine mechanisms rather than mimic physiological conditions. In the current study, CLA did not appear to displace LA in either the PC or PE fractions. However, effects on other fatty acids (e.g. AA in the PC fraction) and the different effects within various fractions (e.g. different levels of incorporation of CLA in PC versus PE) demand attention in future studies to determine potential effects on fatty acid metabolism.

C) Tumor Cell Adhesion

The MDA-MB 231 cell line has been used previously to study the effects of fatty acids on adhesion. For example, Johanning and Lin (1995) demonstrated that 0.75 µg/mL LA (approximately 3 µM) resulted in enhanced adhesion of MDA-MB 231 to collagen IV, fibronectin and Matrigel® compared to cells in media alone. However, these researchers also noted enhanced adhesion to collagen IV and Matrigel® when 2.5 µg/mL EPA (approximately 8 µM) was added. They suggested that these two fatty acids, which exert opposite effects on tumor growth, might act at different sites to enhance or reduce metastasis. For example, enhanced adhesion by LA might act at the secondary site to increase adhesion of tumor cells to the basement membrane and facilitate metastatic spread. In contrast, EPA might act at the primary tumor site to increase adhesion of tumor cells to the basement membrane and reduce the ability of tumor cells to metastasize. Subsequently, German and Johanning (1997) used this cell line to elucidate the effects of EPA at higher concentrations similar to EPA plasma levels found in individuals consuming a high fish oil diet (4.5 µg/mL). They noted a decrease in tumor cell adhesion to collagen IV, fibronectin, and Matrigel® with EPA + media compared to cells in media alone. Finally, in examining graded concentrations of EPA on cell adhesion, Johanning (1996) found that lower concentrations, similar to those used by Johanning and Lin (1995) resulted in enhanced adhesion while higher concentrations, similar to those used by German and Johanning (1997) led to poorer adhesion. Although the results are somewhat confusing, clearly these earlier studies demonstrate that fatty acids *in vitro* can influence adhesion and they suggest that these effects may be concentration dependent.

No previous studies have attempted to measure the effects of CLA on tumor cell adhesion. Therefore, the present study provided novel information regarding the effects of CLA on tumor cell adhesion as a potential anticarcinogenic and anti-metastatic mechanism. In our study, adhesion to various components of the basement membrane was measured. Specifically, adhesion to collagen IV, fibronectin and Matrigel® were examined. Matrigel is a soluble extract from the Engelbreth-Holm-Swarm mouse tumor consisting largely of laminin, collagen IV, heparan sulfate proteoglycan and entactin (Vukicevic, 1992). For all three substrates studied, no significant differences in tumor cell adhesion (***Figures 4.8, 4.9*** and ***4.10***) were noted when cells were exposed to levels of fatty acids which altered cell growth (***Figures 4.6*** and ***4.7***). Whereas Johanning and Lin (1995) noted increases in adhesion with addition of LA to the media compared to cells in media alone, we did not see any differences in adhesion between these groups (LA60 or LA120 versus Cells Alone) for any of the substrates studied. This may have been due to the fact that we used higher LA levels which, in turn, may have resulted in different effects on adhesion, similar to the findings of Johanning (1996). In future studies to continue this work, it would be appropriate to examine the cell adhesive effects of LA and LA+CLA over a range of concentrations to determine whether adhesive function can indeed be modulated depending upon fatty acid concentration. However, from the present study, it was determined that, at a CLA concentration which inhibited cell proliferation and facilitated extremely high membrane incorporation, tumor cell adhesion to components of the basement membrane was not altered. This suggests that it may not be a primary mechanism in reducing tumorigenesis and metastatic spread.

D) Tumor Cell Integrin Expression

Gui *et al.* (1995) suggested that adhesive properties of breast cancer cells might be predicted by cell surface integrin expression. Integrins are a group of transmembrane heterodimeric (α/β chain) glycoproteins and are believed to be the primary mediators of cell-to-substrate adhesion (Johanning, 1996; Hynes, 1992). Integrins bind to various extracellular matrix proteins including collagen IV, fibronectin, and laminin. They also associate with certain cytoskeletal proteins and, therefore, it has been suggested that they may have roles in signalling pathways as well (Schwartz, 1993).

The β_1 chain may associate with a number of α subunits and its ligands include collagens, fibronectin, laminin, VCAM-1 and vitronectin (reviewed by Hynes, 1992). These integrins are widespread on a number of cell types including keratinocytes, colon carcinoma, and mammary epithelia (Sheppard, 1996). Specifically, they have been shown to be moderately expressed by MDA-MB 231 (Gui *et al.*, 1995). The β_4 chain has only been found to associate with the α_6 subunit and its ligand is thought to be laminin although this remains to be established (Hynes, 1992). The $\alpha_6\beta_4$ integrin is less abundant on the cell surface and is localized to the hemidesmosome region to promote adhesion between the basal surface of epithelial cells to the basal membrane (Sheppard, 1996). Although the specific actions of $\alpha_6\beta_4$ in mammary carcinogenesis require further elucidation, the expression of $\alpha_6\beta_4$ has been shown to be altered in several carcinomas and it is thought to be related to tumor progression, invasion, metastasis and apoptosis, perhaps through signalling pathways (Rabinovitz and Mercurio, 1996). This particular integrin is also expressed by the MDA-MB 231 cell line (Jones *et al.*, 1997).

Adhesion molecule expression has been demonstrated to be modifiable by various PUFA and their metabolites. Two groups have noted effects on expression of certain β_3 integrins by human tumor cells in the presence of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a metabolite of arachidonic acid (Chiang *et al.*, 1994; Tang *et al.*, 1994). Also, Jiang and colleagues (1995) found that E-cadherin expression on a variety of human tumor cells, including breast cancer cells, was modifiable by γ-linolenic acid (γ-LNA). Specifically, 100 μM γ-LNA increased E-cadherin expression and reduced tumor invasiveness as measured by *in vitro* assay. EPA and DHA have been shown to modulate the expression of various MHC Class II molecules (molecules which present exogenous antigen) as well as ICAM-1 (an intercellular adhesion molecule) on human blood monocytes after 48 hours of incubation with the fatty acid (Hughes *et al.*, 1996). When these monocytes were activated by IFN-γ, EPA and DHA (66 and 61 μM, respectively) incubation resulted in reduced expression of various MHC Class II molecules as well as ICAM-1. Because these molecules are crucial to the development of an immune response, their reduced expression suggests a mechanism whereby ω-3 fatty acids may suppress immune function. Although studies exist to demonstrate the ability of ω-3 fatty acids to alter adhesion molecule expression on cells such as lymphocytes, there is little information regarding their ability to alter adhesion molecules on tumor cells.

The ability of LA, CLA, or a combination thereof, to alter integrin expression on tumor cells has not been previously examined. Because of the potential modulatory effects of PUFA on tumor cell adhesion, and due to the involvement of integrins in effecting cell adhesion, this seemed a logical investigative interest.

Despite the changes in proliferation (***Figures 4.6*** and ***4.7***) and the incorporation of the incubated fatty acids into the PC (***Table 4.2***) and PE (***Table 4.3***) fractions, the present study demonstrated no effects on the cellular density of either CD29 (β_1 integrin subunit) or CD104 (β_4 integrin subunit; ***Table 4.1***). These results are consistent with our adhesion function (***Figures 4.8, 4.9 and 4.10***) results. Our results suggest that altered expression of integrins is not likely a mechanism of fatty acids, specifically LA and CLA, in modulating metastatic spread of mammary cancer. Despite large differences between LA and CLA on tumor growth and metastasis, the effects of these fatty acids on adhesive function and integrin expression were the same. Interestingly, however, we saw a general reduction in cellular density of CD104 in MDA-MB 231 cells which had been incubated with fatty acids versus those which were cultured in media alone. This may support the general premise that PUFA alter integrin expression, however, as was seen in the present study, different PUFA (i.e. LA and CLA) may have similar effects.

V. Summary

CLA (60 μM) resulted in inhibitory effects on the proliferation of MDA-MB 231 tumor cells. These inhibitory effects were found to be independent of reduced levels of LA or of cytotoxic levels of fatty acids. Despite these effects on tumor cell growth and the high level of incorporation of CLA into both the PC and PE fractions, CLA was not found to affect tumor cell adhesion to extracellular matrix components. Specifically collagen IV and fibronectin, as well as Matrigel® were examined. In addition, no effects of CLA on the expression of adhesion molecules (β_1 and β_4 integrins) were noted. The lack of effect on both adhesive function and adhesion molecule expression suggests that alterations in the interactions between tumor cells and the extracellular matrix are not likely a mechanism in the reduction of tumor metastasis by CLA. However, the degree of incorporation of this fatty acid into tumor cell PC and PE strongly suggest its importance. CLA may act via other membrane effects such as changes in fluidity, ion permeability, enzyme or receptor function, gene expression, or second messenger pathways but this remains to be elucidated. CLA incorporation did not displace LA in either PC or PE similar to findings in mammary tissue (Ip *et al.*, 1996; Ip *et al.*, 1994) and other tissues (Liew *et al.*, 1995; Ha *et al.*, 1990). However, the lower levels of AA and certain ω-3 fatty acids noted in the PC fraction suggest potential alterations in fatty acid metabolism and merit further investigation. Some studies have begun to examine such possibilities and have determined that CLA may affect both the lipoxygenase (Cunningham *et al.*, 1997) and cyclooxygenase (Liu and Belury, 1997) pathways. Also, the preferential incorporation of the *t*10*c*12 isoform seen in the PE fraction contrasts previous studies and provides further impetus to elucidate the biologically active isomer(s). Future research, particularly if individual radiolabelled isomers can be produced, should continue to examine altered fatty acid metabolism, competition for metabolic enzymes, the production of novel bioactive metabolites from CLA and the elucidation of biologically active isomers.

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Chapter Five- General Summary and Discussion

I. General Summary

A) Study One

It was hypothesized that:

i) CLA would enhance anti-cancer immune defenses when fed in diets that were representative of current recommended human dietary patterns.

Positive results were obtained in that CLA enhanced a measure of cell-mediated immunity (T cell proliferation).

ii) CLA would be incorporated into tissue and membrane lipids.

CLA was incorporated into adipose tissue lipids. CLA in the diet did not reduce food intake or affect body weight. It did, however, modulate body composition, but only when fed in a high P/S diet. In these animals, the previously reported reduction in body lipid was seen, This change in lipid composition appeared to be due to increases in body water rather than affecting body protein. CLA was incorporated in the PC and PE fractions of immune cell membranes, but only when fed in a low P/S diet. Incorporation of CLA into membranes and adipose TG appeared to be at the expense of LA incorporation.. Incorporation in adipose TG resulted in a shift in isomer proportions with a relative increase in the *c9t*11 isoform and a relative decrease in the *t*10*c*12 isoform.

iii) the effects of CLA on immune function and tissue incoporation would be modulated by the dietary P/S ratio with low P/S diets augmenting the effects on immune function compared to high P/S diet.

The immune response and tissue incorporation of CLA was influenced by the dietary P/S ratio. T cell proliferation was significantly greater in the diet group which was fed CLA in a low P/S diet. In addition, between the two low P/S diet treatments, CLA supplementation significantly increased the proportion of immune cells which were activated after mitogen stimulation for 48 hours. This increase in activation was not observed with CLA supplementation into the high P/S diets. The effects of CLA on body composition were only seen in animals fed high P/S diets. Dietary P/S ratio also affected the total and isomeric CLA incorporation into adipose stores and immune cell membranes. CLA fed in a low P/S diet resulted in approximately two-fold greater incorporation of total CLA into adipose TG. Despite altering the amount of CLA incorporated into adipose TG, the dietary P/S ratio did not affect ratio of the major isomers incorporated. The effects of diet on CLA incorporation were also seen in the immune membrane phospholipids. Immune cell PC and PE demonstrated enhanced CLA incorporation for rats fed a low P/S diet. When CLA was supplemented into a high P/S diet, levels of CLA in immune PC and PE were similar to that in rats not fed CLA.

iv) dietary P/S ratio would influence immune function.

The low P/S diets enhanced immune function compared to high P/S diets. Our study found that T cell proliferation was significantly enhanced in cells from rats fed a low P/S diet compared to a high P/S diet.

B) Study Two

It was hypothesized that:

i) the addition of CLA to tumor cell cultures would reduce tumor cell proliferation relative to controls.

This hypothesis was supported as adding CLA to cells cultured with LA resulted in a lower proliferative rate, compared to cells cultured with LA alone.

ii) CLA would be readily incorporated into tumor cell membrane lipids.

Incubating tumor cells with CLA resulted in 33% and 16% w/w incorporation into the PC and PE fractions, respectively. The incorporation of CLA did not appear to be at the expense of LA or LNA. However, other fatty acids such as AA and DHA were reduced in the PC fraction, suggesting possible competition with other fatty acids for metabolic pathways. No changes in ω-6 or ω-3 fatty acids were noted in the PE fraction.

iii) CLA would reduce adhesion of tumor cells to various basement membrane components as well as reduce the expression of integrins on the tumor cell surface.

This hypothesis was not supported by the present study. CLA did not alter adhesive function or integrin expression on a mammary tumor cell line, despite reducing cell proliferation.

II. Integration of the Diet Study and Cell Culture Work

Both of the studies described in this thesis examined the incorporation of CLA into cell lipids. In the diet study, adipose TG as well as immune cell membrane PL fractions were examined. In the cell culture work, CLA incorporation into tumor cell membrane PL fractions was examined. In both studies, CLA treatment resulted in CLA incorporation. Interestingly, however, the degree of incorporation varied between the tissues and fractions studied. CLA was incorporated into adipose TG at a similar concentration as those fed in the diet. Dietary CLA accounted for approximately 6% (w/w) of dietary lipid and was incorporated into adipose TG at about 2.9% and 5.7% (w/w) for high and low P/S diets, respectively. Rats fed low P/S diet, but not high P/S diet, demonstrated an increase in CLA in immune cell PC (0.71%and 0.33% for low and high P/S diets, respectively), and PE (0.54% and 0.32% for low and high P/S diets, respectively). Incorporation of CLA into tumor cell membranes *in vitro* was very high. Exposure to 60 μM LA and CLA resulted in 33% (w/w) CLA in the PC fraction and 16% (w/w) in the PE fraction.

In our diet study, CLA was incorporated differently in diets with different fat composition. This strongly suggests competition between CLA and other fatty acids. Comparison of the two low P/S diets indicated that this competition might exist between CLA and LA. Further evidence of this was found in the immune cell PC fraction. In this fraction, comparing the two low P/S diets, levels of LA were reduced when CLA was added to the diet. No effects on membrane LA were noted in the PE fraction, however.

Although not found in every membrane fraction, this study provided strong evidence for competition between CLA and LA. This was supported by the increased levels of CLA found in the animals fed CLA in a low P/S diet, but also by the reductions in LA seen in these same animals in both adipose TG and immune cell PC. In contrast, our *in vitro* study did not provide clear evidence for competition between CLA and LA as levels of LA were unaffected by CLA treatment. CLA did not alter any ω-6 or ω-3 fatty acids in the PE fraction. The possibility of competition between CLA and other fatty acids can not be eliminated however, as reductions in levels of AA and DHA were seen.

Finally, these two studies both examined the incorporation of individual isomers, specifically the *c*9*t*11 and the *t*10*c*12 isoforms. When CLA was supplemented in either a high or low P/S diet, the ratio between these two isomers increased from a dietary ratio of 1.0 to a ratio of approximately 1.7 in adipose TG. This is consistent with the suggestion in the literature that *c*9*t*11 is the biologically active isomer, at least in the hosts' tissues. In contrast, when CLA was added *in vitro* to tumor cells, the ratio between these two isomers was unchanged in the PC (ratio=1.0) and decreased in the PE fraction (ratio=0.7). This is novel information and may call us to re-examine the belief that *c*9*t*11 is the sole biologically active isoform and suggests that the mechanism in the tumor might differ from the host.

Therefore, several conclusions can be drawn regarding the incorporation of CLA in the body. Firstly, the degree of incorporation of total CLA appears to vary between tissues (e.g. adipose, immune cells and tumor cells) and between specific lipid fractions (e.g. PC and PE in *in vitro* study). Secondly, the presence of competition between CLA and other fatty acids may also vary depending on the tissue (e.g. adipose TG versus immune cell PE) and the lipid fraction examined (e.g. PC and PE in *in vitro* study). Specifically, CLA appeared to affect levels of different fatty acids in different tissues and lipid fractions. Finally, the incorporation of individual isomers appeared to vary depending on the tissue (e.g. adipose TG versus tumor cell membrane PC and PE) and the lipid fraction examined (e.g. PC and PE in *in vitro* study).

III. Directions for Future Research

In retrospect, the major limitation of the diet study design was the failure to assess dietary lipid absorption and fecal excretion. Dietary P/S ratio significantly affected body composition, CLA incorporation into adipose, and at least one measure of immune function. Although different fats are known to be absorbed at different levels (Lien et al., 1997), the effects that these differences might have on the absorption and efficacy of CLA are not known. For example, the beef tallow used to increase saturated fats in the diet is less well absorbed than the safflower oil used to increase PUFA (Lien et al., 1997). Therefore, animals fed the low P/S diet likely absorbed less energy from their diet than those fed the high P/S diet. This likely contributed to the observed differences in body weight, as previously discussed in Chapter 3. Differences in energy intake may also have affected the biological efficacy of CLA. Therefore the effects of P/S ratio can not be separated from from possible effects of altered energy intake in this study. Future diet studies should consider ways of ensuring isoenergetic conditions and/or to measure lipid absorption and fecal excretion to determine whether this might affect the incorporation of CLA in the body and its subsequent effects.

Using standard methodologies, the cell culture work clearly demonstrated that CLA incorporation into membrane phospholipids does not influence adhesion to components of the ECM. However, our study only examined two of the major integrins (β_1 and β_4). Before effects on cell adhesion can be ruled out completely as the mechanism for the anti-cancer effects of CLA, it will require examination of other β integrins (e.g. β_3) and the expression of other adhesion molecule subunits (i.e. α_2, α_3) involved in cancer (Pignatelli *et al.*, 1991) which combine with β subunits to make functional integrins. As well, other cell adhesion molecules, including those involved in cell-cell interactions (e.g. E-cadherin, I-CAM1) should be examined. The possibility that CLA may alter tumor cell- matrix interactions can not yet be eliminated however. It may prove beneficial to study the effects of CLA on type IV collagenase activity and on other proteolytic enzymes which affect tumor invasiveness and metastasis. The effect of EPA on type IV collagenase activity has previously been studied with positive results (Liu and Rose, 1995).

Future studies may also wish to explore possible effects of CLA on the expression of adhesion molecules on immune cells. This has been examined with ω-3 fatty acids with positive results (Hughes *et al.*, 1996). Because intimate contact and adhesion between immune cells is vital to immune response (e.g. to provide appropriate signals for proliferation of T cells), this may be a mechanism of CLA in enhancing immunoproliferation.

Finally, future studies should continue to explore the possible effects of CLA on fatty acid metabolism and its potential competition with LA. The diet study strongly suggested competition between CLA and LA whereas the *in vitro* tumor studies did not convincingly demonstrate this. This suggests that the mechanism in the host's tissues and in the tumor may be different. Both studies did reveal alterations in key fatty acids (e.g. AA) which require further exploration. The ability to isolate individual isomers in sufficient quantities and to radiolabel these isomers will prove invaluable in elucidating the metabolic fates of CLA as well as its influences on the metabolism of other fatty acids.

IV. Practical Implications

The actions of CLA as an anticarcinogen are intriguing when one considers that LA, which is similar in structure to CLA, has been shown to enhance mammary tumor formation in rats over a wide range of concentrations (Ip *et al.*, 1985). CLA, however, has been reported to exert its effects at concentrations of 0.1% (w/w), low levels which might reasonably be achieved in the diet (Ip *et al.*, 1991). Other dietary fats have been found to have anticarcinogenic properties, however much higher dietary levels are required to produce a similar effect. For example, EPA, an omega-3 fatty acid, exerts its effects at approximately 10% (w/w) or higher (Reddy *et al.*, 1991).

Thus, CLA is of interest as an anticarcinogen for a number of reasons. Dietary fat has been implicated in increasing cancer risk (Prentice *et al.*, 1988; Schatzkin *et al.*, 1989), yet CLA reduces tumor development in animal models. These actions are in direct contrast to the tumor-enhancing actions of LA. In addition, CLA is derived from animal sources whereas most anticarcinogens (ex. vitamin C, β-carotene, vitamin E) are plant-derived. As well, CLA exists in highest quantities in the very foods that the public has been advised by many groups to decrease due to saturated fat (Health and Welfare Canada, 1990; American Institute for Cancer Research, 1997). The American Institute for Cancer Research recently released diet and health guidelines (1997) to reduce the risk of developing cancer. Included in these were the recommendations to limit red meat consumption to less than 80 g/day if eaten at all, and to limit consumption of fatty foods, particularly those of animal origin. By following such recommendations, consumption of CLA would be extremely low and the opportunities to receive benefits from the anticarcinogenic actions of CLA would be minimized. Recommendations regarding PUFA intake were not addressed in these guidelines, likely due to the possible effects of LA on the tumor. The scientific community may wish to re-evaluate cancer recommendations regarding intake of red meat and animal fats in light of the developing research on CLA.

As well, CLA is of interest as an anticarcinogen because it exerts potent anticarcinogenic effects at low levels which might reasonably be achieved in the diet. This could potentially lead to an effective, easily implemented, low-cost means of reducing tumor incidence and growth, both for the individual and general population chemoprevention. Finally, if the mechanisms whereby CLA exerts its effects could be elucidated, it might provide insight into tumor initiation and promotion. This would lead us closer to effective means of cancer prevention.

This was the impetus behind the diet study and cell culture work: to attempt to elucidate potential anticarcinogenic mechanisms of CLA. The results of the diet study suggested that modulation of the immune system might be one mechanism. Specifically, CLA was found to increase proliferation of immune cells which could enhance the ability of the immune system to respond to a stimulus. Tumor antigens might provide such a stimulus. Also, CLA was found to increase the percentage of various immune subpopulations (T cells, B cells, macrophages) which were activated. This would also act to enhance the ability of the immune system to respond to a stimulus because a greater proportion of immune cells would be primed to respond. The most interesting and novel finding from this study is the importance of dietary fat composition in modulating the effects of CLA. Low P/S diets (P/S=0.2) resulted in greater effects of CLA on the immune system compared to high P/S diets (P/S=1.0). Similar findings have been previously noted where reductions in tumor growth and effects on immune function by ω-3 fatty acids were greatest with a low P/S diet (Robinson and Field, unpublished data). Results such as these call us to examine the current dietary fat recommendations. The North American population is being urged to increase their consumption of PUFA (the major form being LA) and to decrease consumption of saturated fat with the current recommendation being a ratio of 1:1 for these fat types (Health and Welfare Canada, 1990). The trend toward high PUFA intake requires re-examination. Because our study on dietary CLA was carried out on healthy animals, the enhanced efficacy of CLA in a low P/S diet may have implications for the general North American population. For healthy individuals, CLA may be more highly incorporated and exert greater effects on immune function (i.e. T cell proliferation) in the context of a low P/S diet. This may prove important in facilitating greater cancer prevention by CLA. However, our findings may have even greater significance for those with cancer. The immunosuppressive effects of PUFA (specifically LA) are fairly well established. Therefore, an individual who is immune-challenged (i.e. an individual with cancer) may be ill-advised to increase their intake of these fatty acids. Also, as previously mentioned, fatty acids that have been shown to enhance immune function (i.e. ω-3 fatty acids, CLA) appear to exert their effects more effectively when PUFA are less abundant. In the case of ω-3 fatty acids, this appears to be due to competition for elongation/desaturation enzymes. The reasons for this with CLA are not established, although our diet study similarly suggests alterations in fatty acid metabolism.

Because individuals with cancer require effective immune responses to fight against the tumor, it is reasonable to re-examine current dietary fat recommendations. To optimize immune function and to provide a diet which increases the efficacy of CLA, higher levels of saturated fats than are currently recommended may be appropriate.

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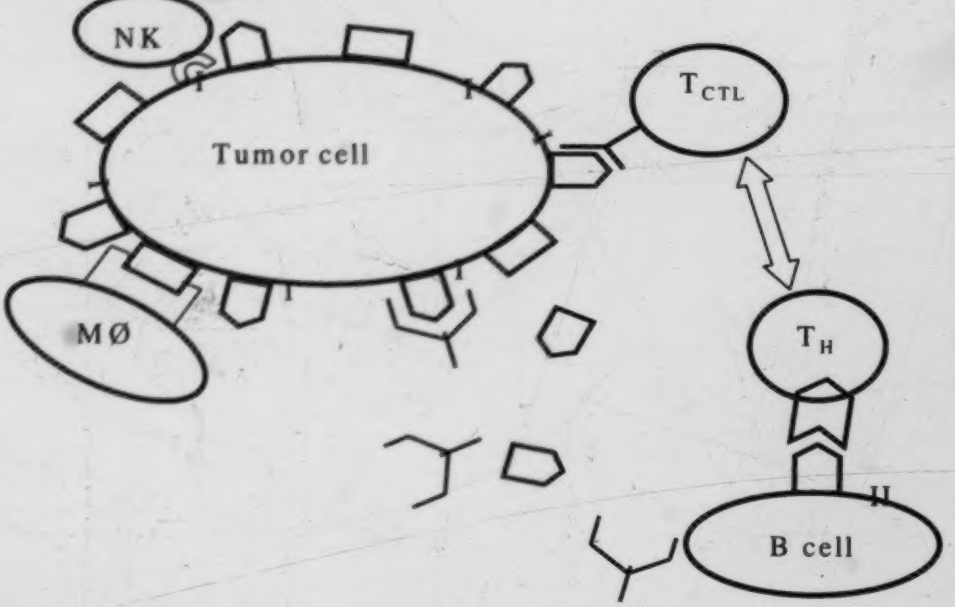
Appendix A. Anti-Tumor Immune Defenses



Immune response may be increased by:

-enhancing tumor MHC Class I expression
-enhancing intracellular antigen processing and loading onto MHC Class I (ex. TAP)
-enhancing accessory molecule expression (ex. B7)
-enhancing immunoproliferation in response to an immune stimulus
-altering cytokine production to enhance immune response (ex. increase in IL-2)
-increasing macrophage, NK, or CTL cytotoxicity
-enhancing tumor-specific antibody production
-altering various immune cell populations (ex. increasing T_H or NK cells)

Tumors may evade immune detection by:

-reducing tumor MHC Class I expression
-inhibiting intracellular antigen processing and loading onto MHC Class I
-producing cytokines that suppress immune response (ex. TGF-β) or alter immune cell populations (ex. increasing suppressor cells)

Appendix B. Diet Compositions for Experiment One

All four diet treatments consisted of 85% (w/w) basal mix (TD 84172, Harlan Teklad, Madison, WI) and 15% (w/w) fat. (Clandinin and Yamashiro. (1980) Effects of basal diet composition on the incidence of dietary fat induced myocardial lesions. *J Nutr* **110**: 1197-1203.)

Basal Mix with Fat Source Omitted (TD 84172)

Ingredient	*Weight (g)*
casein, high protein	286.9
L-methionine	2.7
dextrose, monohydrate	221.5
corn starch	212.5
cellulose	53.1
mineral mix, B-T (170750)	54.0
sodium selenite (0.0445% Na_2SeO_3 in sucrose)	0.3
manganese sulfate $MnSO_4.H_2O$	0.3
vitamin mix, A.O.A.C. (40055)	10.6
inositol	6.6
choline chloride	1.5
	850.0

Although lipid level was constant at 15% (w/w) (150 g/kg diet), lipid compositions for the four diets varied and were confirmed by GC analysis.

Fatty Acid	Diet			
	P/S=1.0 + CLA	*P/S=0.2 + CLA*	*P/S=1.0 - CLA*	*P/S=0.2 - CLA*
Saturates				
12:0	0.1	0.1	0	0.1
14:0	1.4	2.3	1.2	2.3
16:0	14.9	23.4	15.1	23.6
18:0	27.5	52.6	26.9	53.2
20:0	0.6	0.4	0.6	1.0
22:0	0.1	0	0	0.1
Monounsaturates				
16:1 (7)	0.1	0.1	0.1	0
18:1 (9)	7.6	2.7	8.1	3.0
18:1 (7)	0.4	0.2	0.4	0.1
Polyunsaturates				
18:2 (6)	37.8	8.9	45.5	14.3
18:3 (6)	0.1	0.2	0.1	0.2
18:3 (3)	1.1	1.0	1.1	1.0
20:5 (3)	0	0.1	0	0
22:5 (3)	0.2	0	0	0
22:6 (3)	0	0	0	0
CLA	7.0	7.1	0.3	0.2

Table B.1. Fatty Acid Profile of Experimental Diets

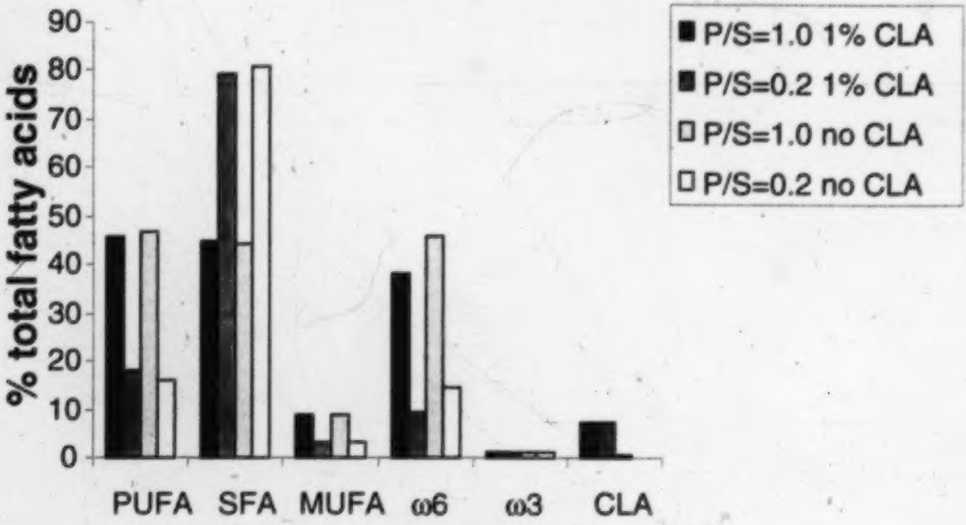


Figure B.1. Major Lipid Types in Experimental Diets

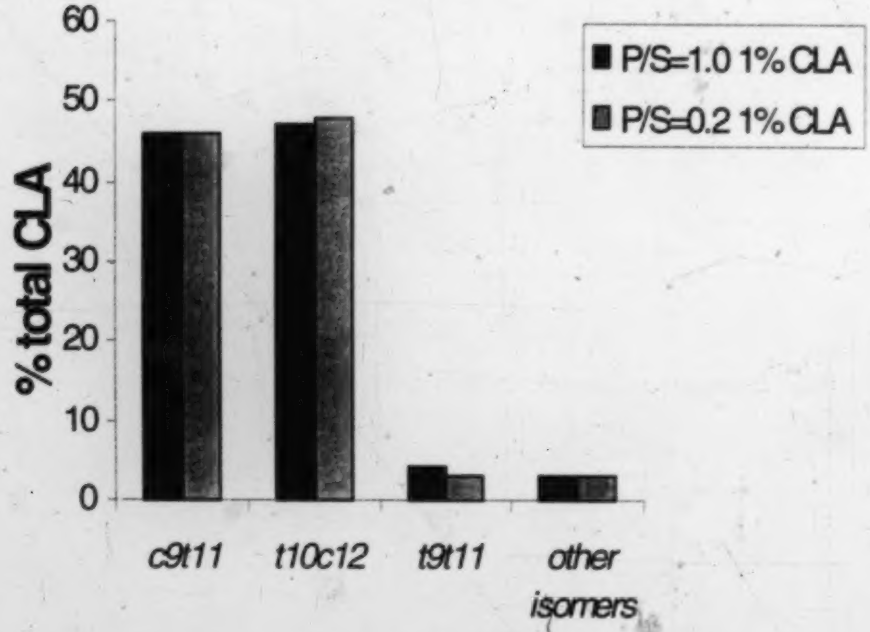


Figure B.2. CLA Isomeric Composition in Supplemented Diets

Appendix C. Compositions of Various Solutions

Splenocyte Lysis Buffer

NH_4Cl	155 mM
EDTA	0.1 mM
$KHCO_3$	10 mM

Make above solution to the following concentrations with ddH_2O. Adjust pH to 7.4.

Solutions for Cytokine (IL-2) ELISA Assay

Binding Solution: 0.1 M Na_2HPO_4 adjusted to pH of 9.0 with HCl.

PBS Solution: 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0 g KCl, q.s. to 10 L, pH to 7.0.

PBS/Tween: Add 0.5 mL of Tween-20 to 1 L PBS.

Blocking Buffer: Add 10% FCS to PBS. Filter before use.

Blocking Buffer/Tween: Add 0.5 mL Tween-20 to 1 L Blocking Buffer.

ABTS Substrate Solution: Add 150 mg 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) to 500 mL of 0.1 M anhydrous citric acid in ddH_2O, pH to 4.35 with NaOH. Aliquot 11 mL per vial and store at -20°C. Add 10 µL 30% H_2O_2 prior to use.

30% H_2O_2: Store in aliquots at -20°C.

Stopping Solution: (20% SDS/50% DMF)- Add 50 mL DMF to 50 mL ddH_2O, then add 20.0 g SDS.

***Standard concentrations used (in pg/mL):** 2000, 1500, 1000, 750, 500, 250, 125, 50, 25, 15.

Solutions for IFN-γ ELISA Assay

Coating Buffer: Dulbecco's Phosphate Buffered Saline (DPBS), pH to 7.4.

Blocking Buffer: DPBS with 1% BSA, 0.25% Tween 20 and 25% heat inactivated FCS.

Wash Buffer: DPBS with 0.05% Tween 20.

Substrate: Tetramethylbenzidine (TMB) and hydrogen peroxide. Sigma One-Step (Product No. T8540)

Stop Solution: 1 M H_2SO_4

***Standard concentrations used (in pg/mL):** 1620, 540, 180, 60, 20.

Solutions for TNF-α ELISA Assay

PBS: 8.00 g/L NaCl, 0.20g/L KCl, 1.15 g/L Na_2HPO_4 and 0.20 g/L KH_2PO_4, all in ddH_2O; pH to 7.4.

Wash Buffer: 0.05% Tween 20 in PBS, pH to 7.4.

Diluent: 0.1% BSA, 0.05% Tween 20 in Tris-buffered saline, pH to 7.3

ABTS Substrate Solution: Add 150 mg 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) to 500 mL of 0.1 M anhydrous citric acid in ddH_2O, pH to 4.35 with NaOH. Aliquot 11 mL per vial and store at -20°C. Add 10 µL 30% H_2O_2 prior to use.

30% H_2O_2: Store in aliquots at -20°C.

Stopping Solution: (20% SDS/50% DMF)- Add 50 mL DMF to 50 mL ddH_2O, then add 20.0 g SDS.

***Standard concentrations used (in pg/mL):** 2000, 1000, 750, 500, 250, 125, 100, 50, 30, 15.

Appendix D. Calculations to Determine Carcass Body Composition

Column	Calculation
A	Final animal weight
B	% protein (determined from freeze dried sample)
C	% lipid (determined from freeze dried sample)
D	% dry matter (determined from freeze dried sample)
E	% ash (determined from freeze dried sample)
F	weight of pressure cooking pot
G	weight of pressure cooking pot + H_2O
H	weight of pressure cooking pot + H_2O + animal carcass (prior to cooking)
I	**animal carcass weight (=H-G)**
J	weight of pressure cooking pot + H_2O + animal carcass (after cooking)
K	**weight of H_2O + animal carcass (after cooking) (=J-F)**
L	**% of start (= K/I*100)**
M	weight of tub used to freeze dry sample
N	weight of tub + homogenized cooked sample (pre-freeze dried)
O	**homogenized cooked sample (pre-freeze dried) (=N-M)**
P	weight of tub + homogenized cooked sample (freeze dried)
Q	**homogenized cooked sample (=P-M)**
R	**% DM of homogenized cooked sample (=Q/O*100)**
S	**100% dry of animal (=I*(L/100)*(R/100)*(D/100)**
T	**% DM of animal (=S/I)*100**
U	**% H_2O of animal (=100 - T)**
V	**weight of H_2O (=I*(U/100))**
W	**% protein of animal (=B/100*T)**
X	**weight of protein (=I*W/100)**
Y	**% lipid of animal (=C/100*T)**
Z	**weight of lipid (=I*Y/100)**
AA	**% ash of animal (E/100*T)**
AB	**weight of ash (=I*AA/100)**
AC	**% of total (=U+W+Y+AA)**
AD	**total weight (=V+X+Z+AB)**

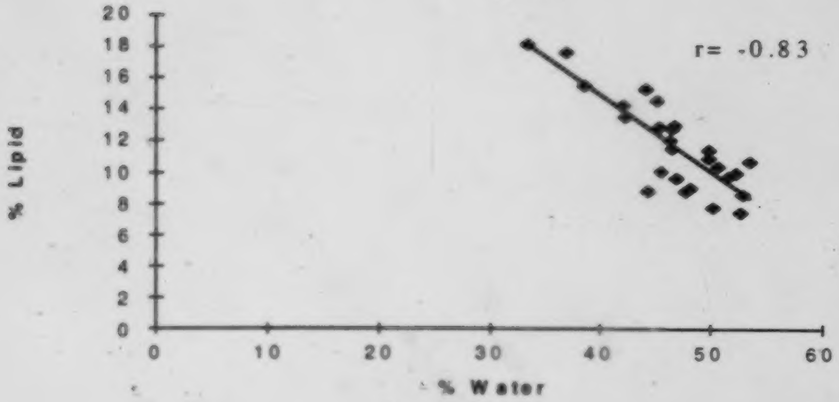


Figure D.1. Percent of Body Water Versus Percent of Body Lipid for Male Rats

The strong correlation between body water and body lipid (***Figure D.1***) indicates the importance of expressing body composition on a whole body basis rather than a dry matter basis. Removing body water from calculations would only be justified if the proportion of body water was similar in all animals. The different results obtained by the two methods is further illustrated by the sample calculations below. In both calculations, expressing values on a dry weight basis equalizes values which are different on a whole body basis.

Sample Calculation of Body Composition

%	Rat 1	Rat 2
Water	50	30
Lipid	30	40
Protein	18	25
Ash	2	5

Lipid

	Dry Weight Basis (%)	Whole Body Basis (%)
Rat 1	60	30
Rat 2	57	40

Protein

	Dry Weight Basis (%)	Whole Body Basis (%)
Rat 1	36	18
Rat 2	36	25

Appendix E. Rationale for Study Design of Cell Culture Work

In choosing an appropriate control, it was thought that the supplementation of a fatty acid that was neutral in affecting proliferation would be appropriate. However, a fatty acid typically chosen as a control (C16:0, palmitic acid) demonstrated potent inhibitory effects on cell growth. This is demonstrated in ***Figure E.1***.

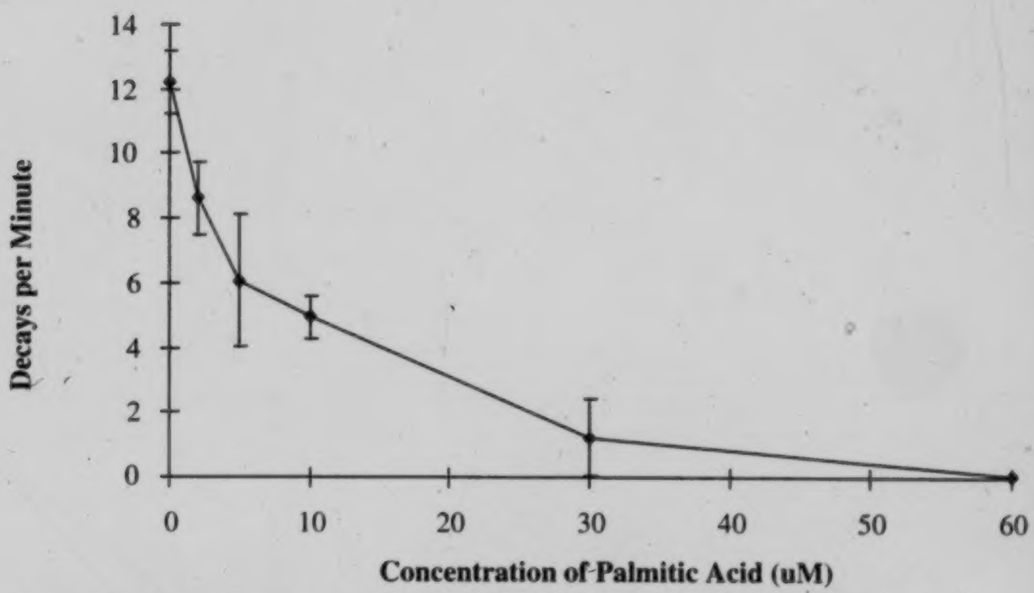


Figure E.1. Concentration Curve for the Effects of Palmitic Acid on Proliferation of MDA-MB-231 Tumor Cells. *Values represent the mean ± SEM, n=3/concentration.*

Therefore, it was thought that linoleic acid (LA) might be appropriate. It is known that LA is essential for tumor growth (Ip, 1987) and can stimulate tumor growth and development (Rose *et al.*, 1994). Because we wished to examine a CLA concentration which was similar to previous studies (Shultz *et al.*, 1992, 1993), we examined the effects of LA at a similar concentration to ensure stimulatory effects. The results are shown in ***Figure E.2.***

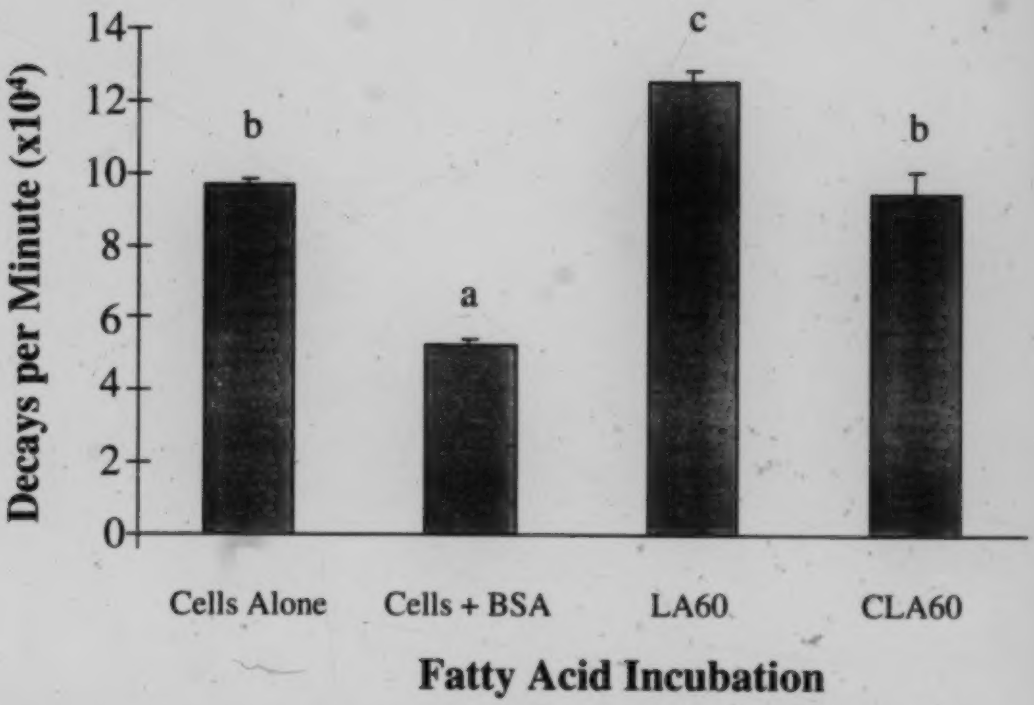


Figure E.2. Proliferation of MDA-MB 231 Cells in Various Cell Culture Conditions. *Cells Alone= media and 1% FCS; Cells + BSA= media, 1% FCS and 0.1% BSA; LA60= media,0.1% BSA and 60 µM LA; CLA60= media, 0.1% BSA and 60 µM CLA. Bars represent the mean ± SEM, n=6/treatment. Bars with different letters are significantly different ($p \leq 0.05$) as determined by one-way ANOVA.*

From these results, the question arose as to whether inhibition of tumor growth with CLA incubation was due to inhibitory actions of this fatty acid or whether it was due to a less than optimal level of LA. It was decided to use a cell culture model in which all cultures contained LA to remove LA deficiency as a possible confounding factor. Any changes in proliferation when LA was replaced by CLA could then be attributed to the actions of CLA.

Finally, the effect of LA on proliferation at various concentrations was examined to determine whether the higher level of LA in our study might prove cytotoxic.

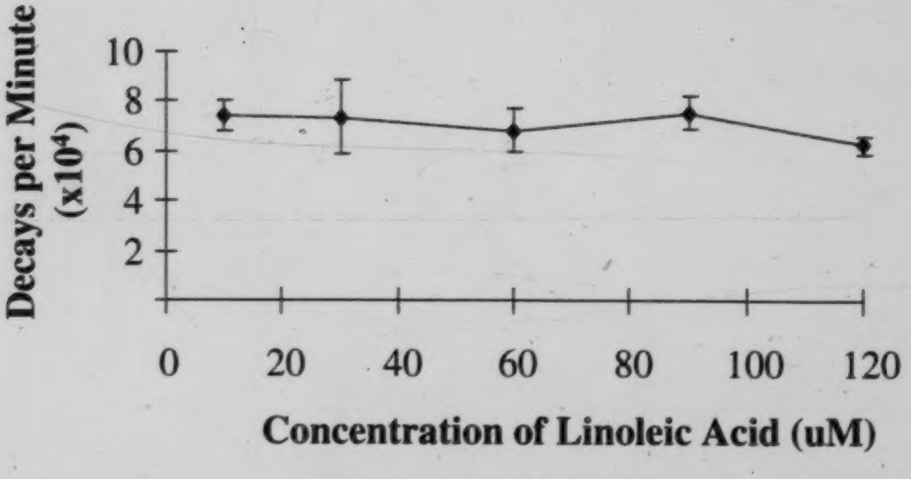


Figure E.3. Concentration Curve for the Effects of Linoleic Acid on Proliferation of MDA-MB-231 Tumor Cells. *Values represent the mean ± SEM, n=3/concentration.*

There were no significant differences in tumor cell proliferation between 60 and 120 µM of LA ($p=0.56$) as determined by one-way ANOVA. Therefore, the 120 µM concentration does not appear to be cytotoxic to the cells. In addition, any reductions or increases in proliferation which are noted in this fatty acid concentration range can be assumed to be due to CLA rather than to changes in concentration of LA.

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